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JACQUES LOEB W. J. V. OSTERHOUT

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Jacques Loeb

Born 1859 · Died 1924

Jacques Loeb, founder of this journal, died in Bermuda, February 11, 1924.

We shall not here attempt to disclose the qualities which quickly made him a recognized leader in biology or the personal traits which endeared him to an ever widening circle of devoted friends. We need only say that his goodness is an open book to all who knew him and his greatness will loom even larger with the lapse of time.

Of his influence on biology it would be difficult to give an adequate account. All his fellow-workers felt its spell and his death will not impair it: many who never knew him will be unconsciously guided by it in ways little guessed.

His conceptions were often so bold and original as to startle conventional thinkers. Fearless in attacking difficult questions of fundamental importance, he showed almost uncanny insight into the most obscure and baffling matters. His discoveries often had a dramatic quality in their unexpected and beautiful solutions of perplexing problems. His results were reached by methods so simple as to compel admiration. His papers were always clear, cogent, and convincing. It is no wonder that he was a powerful stimulus to his fellow-workers.

His mechanistic view-point profoundly affected biology and medicine and had an important influence on psychology and philosophy.

We regret that so little space is here available that the attempt to portray his personality must be left to another occasion. It is possible only to recall some important facts in his career, which to so many has served as example and inspiration.

He was born in Mayen, Germany, April 7, 1859. He attended the Askanisches Gymnasium in Berlin, 1877–1880, the Universities of Berlin, 1880, Munich, 1880–1881, Strassburg, 1881–1885 (M.D., 1884; Staatsexamen, 1885), and in 1885–1886 he was again at the University of Berlin.

He was Assistant in Physiology at the University of Würzburg, 1886–1888, and at the University of Strassburg, 1888–1890. From 1889 to 1891, at the Naples Zoological Station, he laid the foundation for his work on marine biology which was afterward continued at the Marine Biological Laboratory at Woods Hole, Massachusetts, and at Pacific Grove, California. In 1891 he came to America and became Associate in Biology at Bryn Mawr College. From there he went to the University of Chicago as Assistant Professor of Physiology and Experimental Biology in 1892;

later he became Associate Professor, and then Professor of Physiology. In 1902 he accepted the professorship of Physiology at the University of California. In 1910 he became Member of The Rockefeller Institute for Medical Research and remained there until his death.

Attracted in his student days by certain aspects of metaphysics, he became especially interested in problems connected with the freedom of the will. The idea that certain brain functions are localized in definite centers and that human conduct may be profoundly affected by disturbances in these centers led him to study medicine to gain the technical knowledge needed for experiments in order to learn to what extent apparently volitional acts can be controlled by physical and chemical agencies. These studies led him, before the age of thirty, to the revolutionary conception that the actions of animals may be largely explained on a physicochemical basis. He took from the botanist, Julius Sachs, the idea of tropisms and applied it to animals. He sought a mechanistic explanation of animal conduct which should drop the question of purpose and reduce the reactions of animals to quantitative laws. His researches in this field are summarized in English in two volumes, "Comparative Physiology of the Brain and Comparative Psychology" (1900) and "Forced Movements, Tropisms, and Animal Conduct" (1918).

He next undertook to control the growth and form of animals by physical and chemical means. The studies in this field, which he named "Physiological Morphology," covered a very wide range and continued to receive his attention up to the time of his death. The goal at which he aimed was to secure the same degree of control over living matter that the chemist and physicist have over their material and he felt that the best prospect of success lay in applying their methods to biology.

It was characteristic that in the pursuit of these researches he was prompt to utilize recent discoveries in physical chemistry, particularly those connected with the ionic theory and the theory of osmotic pressure. From 1897 on he published papers applying these theories to biological phenomena and they may be said to form the leitmotif of his subsequent work. Out of them arose, almost at once, two important discoveries, antagonism and artificial parthenogenesis. Both resulted from experiments of the simplest kind, carried out on marine organisms.

It was found that the fish, Fundulus, can grow and develop in distilled water but soon dies if sodium chloride is added. The addition of other salts, particularly those of potassium and calcium, which are themselves toxic when they alone are present, produces a harmless solution. Loeb called this a balanced solution, that is, one in which the toxic effects of one substance are offset by the antagonistic action of other substances. This conception proved to be a fruitful one.

As the result of subsequent experiments he concluded that these facts may be accounted for by the effects of the antagonistic substances on the permeability of the protoplasm. It may be added that his experiments on permeability, which covered a wide range, led to the conclusion that Overton's hypothesis is untenable.

His experiments on artificial parthenogenesis may be truly called epoch-making. In 1913 he reviewed them in a volume entitled "Artificial Parthenogenesis and Fertilization" (following the publication of two volumes on this subject in German). He continued work in this field for some years after the publication of this volume. He aimed at a complete analysis of the mechanism of fertilization, development, and heredity. His work on fertilization indicated that the principal function of the sperm in stimulating development is to carry into the egg a substance which produces a surface change leading to the production of the fertilization membrane. This effect could be brought about, independently of sperm, by a variety of physical and chemical agencies.

At the same time he succeeded in finding means to bring about crosses which never occur in nature. By a slight change in the composition of the sea water eggs could be fertilized by sperm of other species which could not normally enter the egg.

In the course of these studies a great number of questions presented themselves which excited his keen interest. The variety and extent of these problems can only be realized by a careful consideration of the contents of the volumes entitled "Studies in General Physiology" (1905), "Dynamics of Living Matter" (1906), "The Mechanistic Conception of Life" (1912), and "The Organism as a Whole" (1916).

Among these subjects may be mentioned the rôle of oxygen in metabolism, toxicity, and development. Another in which he was deeply interested is the cause of natural death and the means of lengthening life. In the course of these studies he found that in certain cases low temperature prolongs life to a remarkable degree. In this connection it may be recalled that the first studies on the temperature coefficient of the heart beat and of the transmission of stimuli in nerves were made by his students.

Another investigation which he initiated, and which grew naturally out of these studies, showed that the electric potentials existing in the organism can, in many cases, be accounted for qualitatively and quantitatively by relatively simple means, and that they can be imitated to a considerable extent by artificial models.

As he truly said, many biologists accept a mechanistic explanation of various functions of the organism but fail to employ mechanistic conceptions in dealing with the larger problems of organization and adaptation. It was, however, precisely these problems which fascinated him and he did not hesitate to attack them from this point of view. He showed that many characteristics of the organism which are regarded as adaptive may be explained on a mechanistic basis. He felt that here, as everywhere in biology, progress requires quantitative investigation and with this in view he began the quantitative studies on regeneration upon which he continued to work until his death. His latest book, "La nature physico-chemique de la régénération" (Paris, 1924), is devoted to this subject.

His work began, as was said, with questions concerning the freedom of the will. But he found that in order to study these he must attack the simpler problems involved in the behavior of lower organisms. These in

turn required for their understanding a study of the physical and chemical reactions on which they are based. It is, therefore, not surprising that in the closing years of his life he came to devote his attention almost wholly to the properties of colloids, upon which life phenomena so largely depend. In a recent volume on "Proteins and the Theory of Colloidal Behavior" (1922) he contends that the behavior of colloids may be explained by the ordinary laws of chemistry without recourse to theories based on adsorption. As in earlier researches he had found a clue to the solution of many problems by applying the theories of electrolytic dissociation and osmosis, so in the work of his later years he discovered a guiding principle in the theory of the Donnan equilibrium. By applying this he was able to give quantitative explanations of some of the most important properties of proteins and to reduce them to simple mathematical laws. These studies, imporant for chemistry as well as for biology, form a fitting termination of his activity.

Thus closed a career rich in the joy of pioneer adventure in fresh fields of thought, abounding in brilliant discoveries, and splendidly stimulating far beyond the boundaries of biology. It will always stand out as a prominent feature of the progress of biology toward the status of an exact science. It is a career which reveals everywhere a creative imagination and capacity found only in minds of the highest order.

IONIC THEORY OF ACTIVITY OF NERVE CENTERS AND OF PROPAGATION OF NERVE IMPULSE.

By P. LASAREFF.

(Received for publication, January 12, 1924.)

In this article I intend to give an explanation of the ionic theory of function of nerve centers in connection with propagation of nerve stimulation. The experiments of many authors and our observations[1] show that during continuous action of nerve centers there takes place a periodic process of stimulation which results in a periodic electromotive force. The periods of different centers are not identical and vary from 0.01 to 1 second.

From the view-point of ionic theory of stimulation, the periodicity of actions of centers must depend on the periodic changes of the concentration of ions in the centers. These changes are brought about by periodic chemical reactions. The kinetics of such a periodic reaction is not well known. The attempt of many authors to develop the doctrine of this reaction, based on the kinetics of ordinary chemical reactions, does not give definite results and if we want to apply the kinetics of periodic reactions to the domain of physiology of the nervous system, it suffices to assume that in the nerve cells during activity there takes place a periodic chemical reaction resulting in a periodic change of ionic concentration.

The equation of this periodic change of concentration C can be written as follows:

$$C = f(t) \tag{1}$$

in which $f(t)$ is a periodic function of time, t.

The simplest case of this function is that in which

$$C = f(t) = C_0(1 + \sin nt) \tag{2}$$

[1] Lasareff, P., Ionentheorie der Reizung, in Lipschütz, A., Sammlung von Abhandlungen aus dem Gebiete der Biologie und Medizin, Bern and Leipsic, 1923.

(n is a frequency, $n = \frac{2\pi}{T}$, T is the period.) In this case C varies between 0 and $2C_0$.

The value of C given by the equation (2) satisfies the following equation of kinetics.

$$\frac{d^2C}{dt^2} = n^2 (C_0 - C) \tag{2a}$$

We can conclude from the equation (2) that every period of this reaction consists of two phases: During the first phase of the period (part *a*) we have a destruction of substance in the nerve and the concentration of C rises (from 0 to $2C_0$); during the second phase (part *b*) complete restitution takes place, when $C = 0$. In other words, in every period we have the time of fatigue (part *a*) and the time of rest (part *b*). If we observe nerve cells in which a periodic reaction takes place, in periods of time $nt = 2\pi A$ in which A is a whole number, we find that C has the constant value C_0 and we can conclude that this center is not fatigued.

The indefatigability and the periodic function are, from this theory, in a close connection. In our paper published recently,[2] we have given a proof of the indefatigability of the visual centers of peripheral vision, predicted by the ionic theory of adaptation developed by us in 1914. It was found that during adaptation when the sensitiveness of the visual apparatus varies 100,000 times, the sensitiveness towards electric stimulation does not vary.

It may be assumed by the analogy in the kinetics of the reactions[3] that also other centers of the brain are not fatigued during their activity. We therefore arrive at the conclusion of the indefatigability of brain centers either by starting from the theory of adaptation or from the general doctrine of periodic action of nerve cells.

In conclusion, it is necessary to point out that the influence of physical and chemical agents upon the course of ordinary periodic reactions is identical with their influence upon the activity of the

[2] Lasareff, P., *Arch. ges. Physiol.*, 1923, cc, 119.

[3] Lasareff, P., Recherches sur la théorie ionique de l'excitation, Edition de l'Institut Scientifique de Moscou, Moscow, 1918.

nerve centers.[4] As an example of this identity, we may take the case of the influence of electric stimulation. In both cases we have a minimal effect when the amplitude of the periodic electrical current, a, and the frequency, n, have the following relation.

$$\frac{a}{\sqrt{n}} = \text{const. (law of Nernst)}$$

Furthermore, the action of temperature on both phenomena is the same. Also, many chemical substances produce an analogous effect on the rate of pulsation of nerve centers and of periodic chemical reactions. Thus, we can conclude that the chemical and the dynamic conditions in active nerve cells are similar to those of periodic chemical reactions.

Stimulation of nerve centers results in a propagation of the stimulus into the nerve. The derivation of the equation for the propagation of nerve impulses can be based on the following experimental facts. First, the velocity of propagation of the stimulus is constant, and second, the form of the electric impulse accompanying the stimulation is not changed during its propagation. We can express these data mathematically if we write that the impulse propagates in the form of a wave. The differential equation of propagation then is

$$\frac{\delta^2 C}{\delta t^2} = v^2 \frac{\delta^2 C}{\delta x^2} \tag{2b}$$

in which v is the velocity of propagation and x the distance from point $x = 0$. The integral of this equation is

$$C = \phi\left(t \pm \frac{x}{v}\right)$$

The function ϕ must satisfy the conditions in the beginning of the coordinates, where we may imagine the nerve cell to be located and where, therefore, there must exist the condition

$$C_1 = C_0 (1 + \sin nt) \tag{3}$$

[4] Kremann, R., Die periodischen Erscheinungen in der Chemie, Sonderausgabe aus der Sammlung chemischer und chemisch-technischer Vorträge, Stuttgart, 1913.

The concentration C in the nerve is obtained if we make

$$C = C_0\left[1 + \sin n\left(t \pm \frac{x}{v}\right)\right] \tag{4}$$

This concentration satisfies the two conditions: the constancy of velocity of propagation and the condition at the beginning of the coordinates given in equation (3).

If C has the character not of a simple sinoidal function but that of a more complicated general periodic function, we can write

$$C_1 = f(t) = C_0 + C_1 \sin(nt + \alpha_1) + C_2 \sin 2(nt + \alpha_2) + \ldots$$

and in this case, we have

$$C = C_0 + C_1 \sin\left[n\left(t \pm \frac{x}{v}\right) + \alpha_1\right] + C_2 \sin 2\left[n\left(t \pm \frac{x}{v}\right) + \alpha_2\right] + \ldots$$

This C satisfies the equation (2b) and the condition at the beginning of the coordinates.

Our assumption is that the chemical reaction in the nerve cell brings about a chemical reaction in the nerve. In order to obtain the equation of the kinetics of this reaction in the nerve, we must assume that in the nerve, the concentration of the sensitive substance is uniform on all points. The relation between $\frac{dC}{dt}$ and C determines the character of this reaction. The assumption of equality of C in all points of the nerve is identical with the one that in the nerve C is a function of t only and does not depend on x. Therefore,

$$\frac{\delta^2 C}{\delta x^2} = 0$$

Then the time variation of C is expressed by the equation

$$\frac{\delta^2 C}{\delta t^2} = 0$$

or

$$\frac{\delta C}{\delta t} = A \tag{5}$$

and

$$C = At + B$$

A and B are constants.

We see from equation (5) that the velocity of reaction is constant and therefore the reaction beginning at the time, t, cannot stop and ends when the sensitive substance in the nerve is entirely decomposed. The stimulation in the nerve from this point of view cannot vary in intensity.

If the stimulus is too small to provoke the stimulation there is no reaction; if, on the other hand, it is sufficient to produce stimulation, then there always results a maximum ionic concentration. In this way we may explain the "all or nothing" law.

INTENSITY DISCRIMINATION AND THE STATIONARY STATE.

By SELIG HECHT.*

(From the Department of Physical Chemistry in the Laboratories of Physiology, Harvard Medical School, Boston.)

(Received for publication, January 4, 1924.)

I.

Most animals easily became adapted to their sensory environment. The initial effect of a stimulating agent is to elicit a characteristic response. If the stimulation is maintained, the organism very quickly comes into sensory equilibrium with it. Again to elicit the characteristic response of the organism, it is necessary now to increase the intensity of the stimulating agent. The extent of this increase varies with the adapting intensity, and may be considered a measure of the animal's sensitivity. Therefore, with each condition of sensory equilibrium, there is associated a degree of sensitivity which corresponds to the magnitude of the environmental agent producing the equilibrium state. The present paper contains a study of this quantitative aspect of sensory adaptation.

II.

1. The clam, *Mya arenaria*, exhibits the features of sensory adaptation in a simple manner. If exposed to light, it responds by the retraction of its photosensitive siphons. If the exposure is continued, no additional response is elicited, and the animal becomes adapted. To produce a response again, it is necessary to increase the intensity of the light. The response which occurs is of the same nature as before, and if the intensity is maintained, is immediately followed by the adaptation of the animal.

Consider this from a slightly different angle. Let *Mya* be adapted to a light of intensity I. A slight increase in this intensity produces

* National Research Fellow.

no response on the part of the animal. However, there can be found an increase in intensity, ΔI, which when added, will just elicit a response. The ratio $\frac{\Delta I}{I}$ may be considered as a measure of the differential sensitivity of *Mya* at the intensity I.

2. The ratio $\frac{\Delta I}{I}$ as a measure of differential sensitivity in human sense organs has had an interesting history, of which only the major aspect is pertinent at this point. Weber (1834) first pointed out that for the sense of touch $\frac{\Delta I}{I}$ seems to possess a constant value regardless of variations in I. Other investigators soon found a similar constancy of $\frac{\Delta I}{I}$ for other senses, particularly for vision. Fechner (1858) after assuming that the ratio $\frac{\Delta I}{I}$ represents a unitary value in sensation (ΔS) integrated the function $\Delta S = k \frac{\Delta I}{I}$ and enunciated his famous psychophysical law that the sensation is proportional to the logarithm of the intensity of the stimulus. It is sufficient merely to add that careful experiments such as those of Koenig and Brodhun (1889) with the human eye have long ago shown that the ratio $\frac{\Delta I}{I}$, far from being constant, varies in a perfectly definite way. At low intensities it is large; at moderate intensities it drops to less than a thirtieth of its original value; and at high intensities it again increases to several times its lowest value.

The theoretical significance of these regular changes in the value of $\frac{\Delta I}{I}$ for the eye is at present unknown. This is not unexpected if we recall that changes in intensity affect not only the two visual mechanisms—the rods and the cones—in the retina, but the pupillary mechanism in the iris as well. Such complications do not exist in the sensory mechanism of *Mya*. The behavior of the ratio $\frac{\Delta I}{I}$ should therefore be amenable to theoretical analysis in terms of what has already been described as the physicochemical nature of the photosensory mechanism (Hecht, 1919–20, *a*).

III.

1. The method of securing the experimental values of $\frac{\Delta I}{I}$ deserves careful consideration. The values of the adapting intensity, I, are secured simply by exposing an animal to a 400 watt, concentrated filament Mazda lamp, the light from which is varied in intensity by the interposition of neutral screens of different absorbing powers. The obvious method of obtaining the discriminable increment ΔI is to determine the minimum increase in intensity which will just elicit a response. There are, however, two objections to this procedure. First, it requires an illumination which can be varied continuously and quantitatively over a large range. This, though difficult, is not impossible. Second, the method involves the judgment of a response which is barely perceptible, and calls for repeated tests of the effectiveness of slightly lower or slightly higher intensities. Since at least 5 minutes must be allowed between tests with *Mya*, the procedure, aside from not being clean cut, becomes very tedious and time-consuming. I therefore adopted a radically different method.

We know that the reaction time of *Mya* to light varies inversely as the logarithm of the intensity (Hecht, 1919–20, *b*). By determining this relationship for an animal adapted to each intensity, we can secure values of ΔI which represent the intensity added to produce a given response in a given reaction time. Thus, instead of recording the discrimination of *Mya* between one intensity and the next barely perceptible one, we can with ease find the added intensity necessary for *Mya* to discriminate with any degree of magnitude, depending on the time required to produce a response. Moreover, the relation between log I and the reaction time is easily established graphically from as few as three points. This makes it possible to carry one animal through a whole series of adaptations in a single day, and still leave half an hour for it to remain in the dark between adaptations.

2. The experimental procedure is then as follows. An animal is exposed to light of a given intensity, the position of the animal and the light being given in Fig. 1. Though it becomes adapted in a few seconds, it is allowed to remain exposed for 15 minutes. At this point the stimulating light of a given intensity is added and the reac-

tion time of the animal measured with a stop-watch. The stimulating light is then immediately shut off, and the animal remains exposed to the adapting light alone for 5 minutes. After this period the stimulating light, but at a different intensity, is again added, and the reaction of time of the animal measured. After another rest of 5 minutes during which the animal again remains exposed to the adapting light only, the stimulation is again repeated but with a still different intensity and the reaction time measured.

After these three readings have been obtained, the animal is removed and kept in the dark for half an hour. It is then exposed to a different adapting intensity, and three readings made of the reaction time

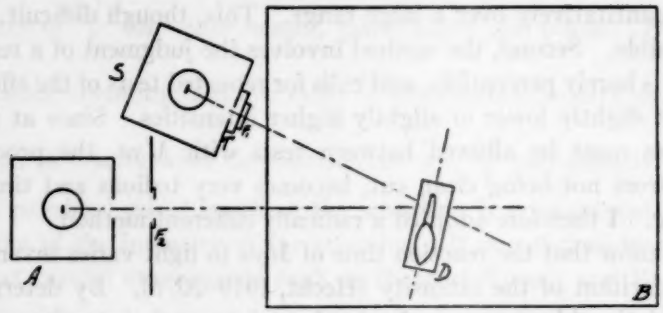

FIG. 1. Arrangement of apparatus. The animal in the dish, *D*, rests on the table, *B*, where it is illuminated by the adapting light, *A*, and by the stimulating light, *S*. The intensity of the light reaching the animal is controlled by filters placed in the holders, F_1 and F_2. Exposure to the stimulating light is effected by the shutter, *T*.

to three intensities of the stimulating light. A half hour in the dark follows, after which a still different adaptation intensity is used. In this way the process is repeated until all the adaptation intensities have been run through. The order in which the adapting intensities are used is purely chance.

3. The intensities of the adapting and stimulating lights are varied by means of neutral filters made from uniformly fogged photographic plates of varying density. After proper mounting they were carefully calibrated photometrically and spectrophotometrically to insure their non-selectivity for any part of the spectrum. I find these filters superior in the latter respect to the "neutral" filters of commerce,

individual examples of which have upon examination been found far from non-selective. The exposure to the stimulating light is controlled by a shutter.

TABLE I.

Relation between Stimulating Intensity and Reaction Time at Different Adaptation Intensities.

Adaptation intensity.	Stimulating intensity.	Reaction time.
meter candles	*meter candles*	*sec.*
0	2.34	2.84
	5.73	2.49
	44.3	2.04
0.574	18.1	2.86
	44.3	2.51
	239.8	2.05
2.38	63.8	2.57
	239.8	2.16
	435.	2.02
8.57	63.8	2.89
	239.8	2.27
	435.	2.09
35.4	120.2	2.87
	239.8	2.44
	1,286.	1.92
73.7	239.8	3.02
	435.	2.39
	1,286.	2.08
412.5	435.	2.95
	1,286.	2.27
	2,836.	2.06
1,100	1,286.	2.98
	2,836.	2.32
	6,950.	2.13

Care must be exercised in the control of the temperature because of the prolonged exposure to strong lights. A constant temperature to within 0.5°C. was maintained by adding cold or warm sea water as

necessary, and by frequent changes of the sea water in the dishes during the half hour dark period. The average temperature for all the final experiments is 19.1°C.

IV.

1. A large number of preliminary experiments[1] were occupied with determining the proper adapting and stimulating intensities required to cover a large range of adaptation illuminations compatible with a

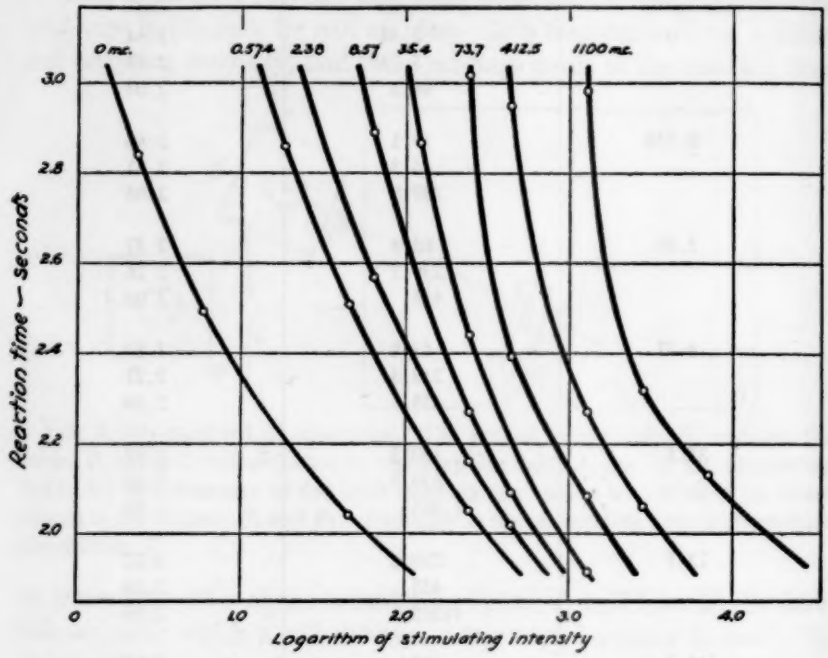

FIG. 2. Data showing the relation between stimulation intensity and reaction time at different adaptation intensities. Each point is the average of ten measurements, one each with ten animals.

convenient range of reactions to the stimulating light. After this, a final series of measurements was made with ten individuals under the chosen conditions of adaptation, stimulation, rest, and temperature.

[1] These experiments were made at the Marine Biological Laboratory at Woods Hole during the summer of 1923. I wish to express my thanks to the Director, Dr. Frank R. Lillie, for placing laboratory space at my disposal.

The results are given in Table I, where the values of the reaction time in the third column are averages of ten measurements, one with each animal. The results are shown graphically in Fig. 2. It is apparent that they are uniform and smooth and are therefore amenable to quantitative analysis.

2. To derive from these data quantities which will describe the differential sensitivity of *Mya*, it is necessary to find values of ΔI which represent the same degree of discrimination at the different adaptation intensities. I have chosen three levels of the reaction time at which to compare ΔI at different values of I, *viz.* 2.1, 2.5, and

TABLE II.

The Discriminable Increment ΔI *and the Ratio* $\frac{\Delta I}{I}$ *for Three Values of the Reaction Time at Different Adaptation Intensities.*

Adaptation intensity.	Reaction time = 2.1 sec.		Reaction time = 2.5 sec.		Reaction time = 2.9 sec.	
	ΔI	$\frac{\Delta I}{I}$	ΔI	$\frac{\Delta I}{I}$	ΔI	$\frac{\Delta I}{I}$
m.c.	*m.c.*		*m.c.*		*m.c.*	
0	29.9	∞	5.48	∞	2.06	∞
0.574	194.1	338.2	45.7	79.6	16.2	28.2
2.38	307.	129.0	77.3	32.4	28.1	11.8
8.57	420.	49.0	132.4	15.4	63.0	7.35
35.4	612.	17.3	210.	5.93	116.9	3.30
73.7	1,084.	14.7	363.	4.93	252.	3.42
412.5	2,355.	5.71	780.	1.89	457.	1.11
1,100.	10,230.	9.30	2,046.	1.86	1,346.	1.22

2.9 seconds, because these represent the closest approach to most of the experimentally determined data. For this purpose I have plotted the experimental results of Table I and Fig. 2, on a large scale and have read off the value of the stimulating intensity (ΔI) from the smooth curves at the three points noted. The results are given in Table II. It is apparent that as the adaptation intensity I increases, the stimulating intensity ΔI which represents a given discrimination by *Mya* also increases.

3. Of interest to us at present, however, is not so much the absolute values of ΔI as their values relative to the adaptation intensity, I,

in other words the ratio $\frac{\Delta I}{I}$. This ratio is therefore also given in Table II. It is apparent at once that $\frac{\Delta I}{I}$ is far from constant. For two closely similar intensities like 35.4 and 73.7 meter candles, the ratio may be considered roughly constant. But over any reasonable

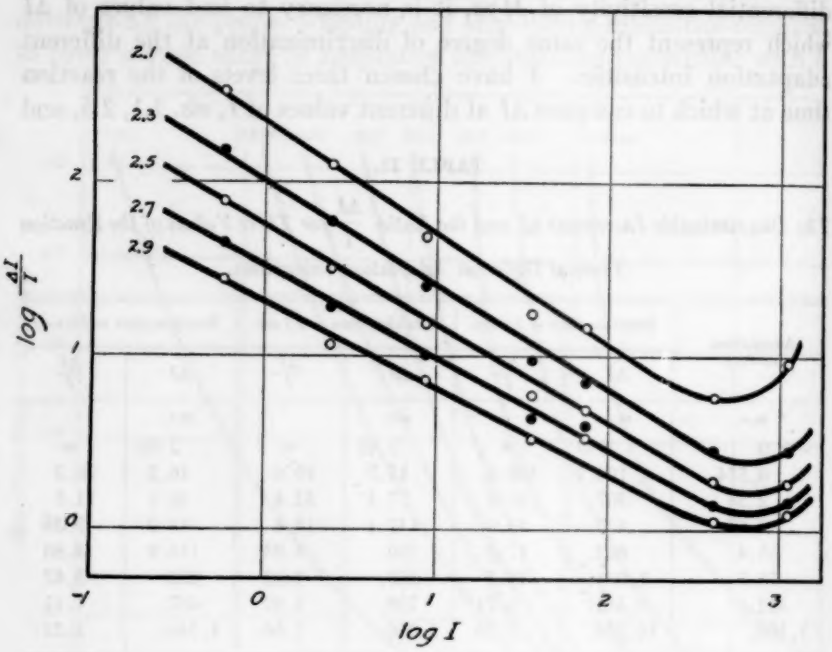

FIG. 3. Curves showing relation between log I and log $\frac{\Delta I}{I}$. It is apparent that the ratio $\frac{\Delta I}{I}$ is never constant, but varies in a definite manner with I, first decreasing and then increasing.

range $\frac{\Delta I}{I}$ shows no constancy. What is more significant, however, is that its mode of variation is regular and the same in all three series. I have determined the values of ΔI and also of $\frac{\Delta I}{I}$ for reaction times of 2.3 and 2.7 seconds as well. These also show the same course of the values of $\frac{\Delta I}{I}$.

In order to demonstrate precisely how this ratio varies, I have plotted its values in Fig. 3, including the data for all five reaction times. To get them all on a single graph and to bring out their similarities, I have plotted $\log \frac{\Delta I}{I}$ instead of the ratio itself. Two things are strikingly apparent from Fig. 3. First, the mode of variation of $\frac{\Delta I}{I}$ is the same for the five sets of data. Hence an adequate analysis of one of them is sufficient for all. Second, there is no tendency of $\frac{\Delta I}{I}$ ever to be constant. As I increases, $\frac{\Delta I}{I}$ steadily decreases to a minimum after which it begins to rise. It follows that if I were further increased, $\frac{\Delta I}{I}$ would increase still more. It was not possible to carry the experiments any further because of the lack of lights of sufficient intensity. Calculation shows that with an adaptation intensity of 3,000 m.c., the stimulating intensity to produce a response in 2.1 seconds would have to be about 80,000 m.c.—an intensity obviously difficult to handle.

4. From these data it must be clear that all notions based on the constancy of $\frac{\Delta I}{I}$, such as the application of the Weber-Fechner law to animal responses, rest on an uncertain foundation and must be viewed with skepticism. The matter, however, cuts even deeper than that. The Weber-Fechner law itself is by no means invulnerable. Koenig and Brodhun's (1889) classic experiments have long ago demonstrated that the ratio $\frac{\Delta I}{I}$ for the eye is not constant. Fig. 4 gives the data of one of their experiments with the effect of white light on Brodhun's eye. The way in which $\frac{\Delta I}{I}$ varies is obvious. To emphasize the significance of the present experiments with *Mya*, I have drawn in Fig. 5 the data taken from Column 3 of Table II showing how $\frac{\Delta I}{I}$ itself (not $\log \frac{\Delta I}{I}$ as in Fig. 3) varies with I. It is only necessary to compare

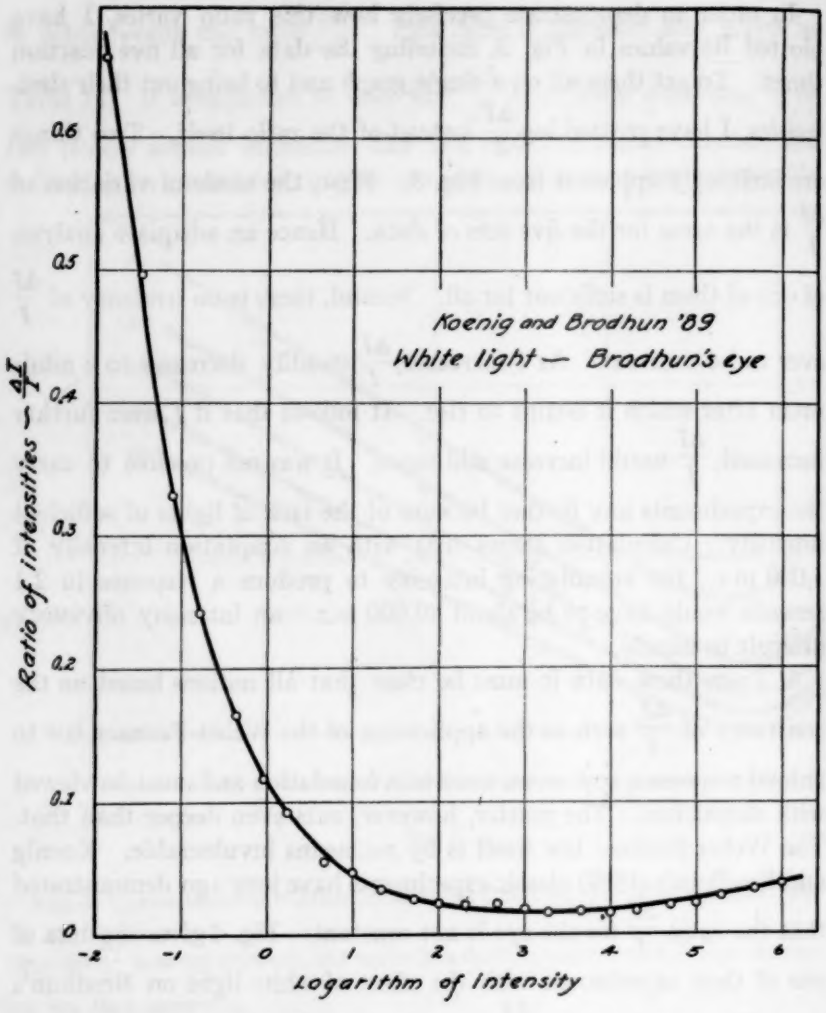

FIG. 4. Relation between $\frac{\Delta I}{I}$ and log I for the human eye. The data are from an experiment by Koenig and Brodhun (1889). Compare this with Fig. 5.

Figs. 4 and 5 to realize the importance of a proper analysis of the data with *Mya* and their consequences for the general physiology of sense organs and other irritable tissues. It is to this analysis that we must turn now.

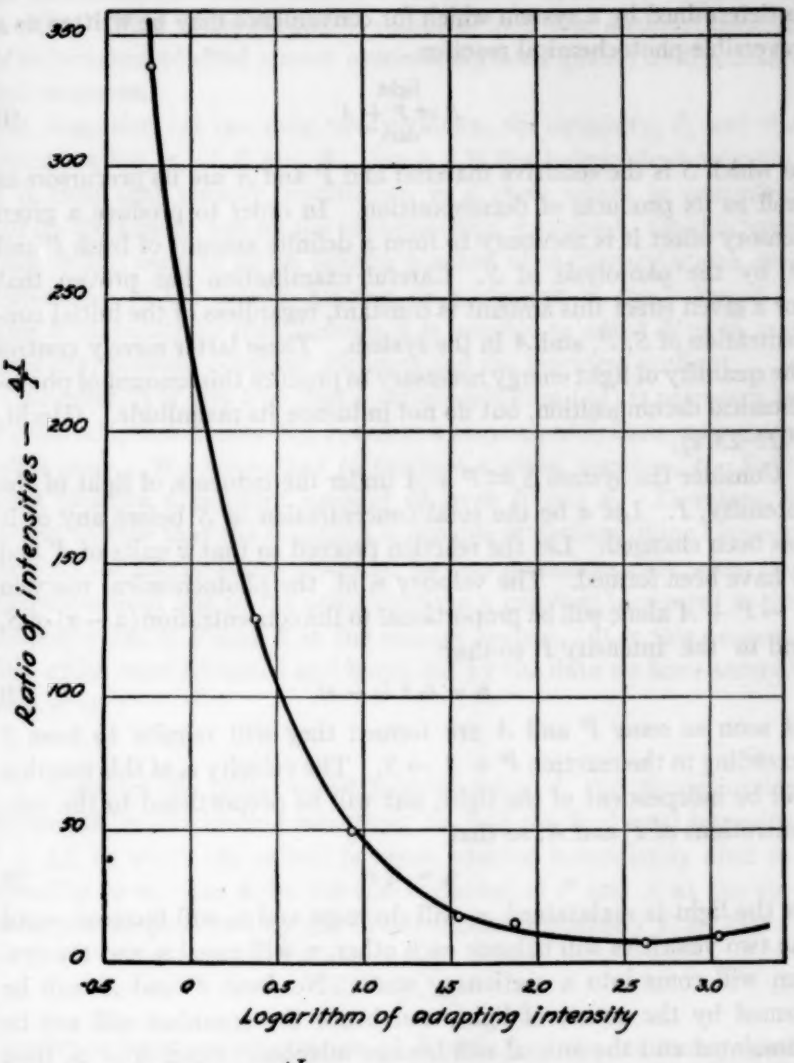

FIG. 5. Relation between $\frac{\Delta I}{I}$ and log I for *Mya*. Comparison with Fig. 4 giving this relation for the human eye shows how essentially similar the two sets of data are.

V.

1. On the basis of varied evidence I have already shown (Hecht, 1919–20, *a*) that the initial event in the reception of light by *Mya*

is determined by a system which for convenience may be written as a reversible photochemical reaction

$$S \underset{\text{"dark"}}{\overset{\text{light}}{\rightleftharpoons}} P + A \tag{1}$$

in which S is the sensitive material and P and A are its precursors as well as its products of decomposition. In order to produce a given sensory effect it is necessary to form a definite amount of fresh P and A by the photolysis of S. Careful examination has proven that for a given effect this amount is constant, regardless of the initial concentration of S, P, and A in the system. These latter merely control the quantity of light energy necessary to produce this amount of photochemical decomposition, but do not influence its magnitude. (Hecht, 1922–23, *c*).

Consider the system $S \rightleftharpoons P + A$ under the influence of light of the intensity, I. Let a be the total concentration of S before any of it has been changed. Let the reaction proceed so that x units of P and A have been formed. The velocity v_1 of the photochemical reaction $S \rightarrow P + A$ alone will be proportional to the concentration $(a - x)$ of S, and to the intensity I, so that

$$v_1 = k_1 I (a - x). \tag{2}$$

As soon as some P and A are formed they will reunite to form S according to the reaction $P + A \rightarrow S$. The velocity v_2 of this reaction will be independent of the light, but will be proportional to the concentrations of P and A, so that

$$v_2 = k_2 x^2. \tag{3}$$

As the light is maintained, v_1 will decrease and v_2 will increase, until the two velocities will balance each other, v_1 will equal v_2 and the system will come into a stationary state. No fresh P and A will be formed by the action of light, and hence the organism will not be stimulated and the animal will become adapted. Since $v_1 = v_2$, then

$$k_1 I (a - x) = k_2 x^2$$

and, writing $\frac{k_1}{k_2} = K$, we get

$$KI = \frac{x^2}{a - x} \tag{4}$$

as the equation of the stationary state underlying sensory equilibrium in *Mya*. This entire process is complete in a few seconds, because *Mya* becomes adapted almost immediately after giving its characteristic response.

2. Equation (4) has only two variables, the intensity, I, and the concentration, x, of P and A. Since I is the independent variable, it is clear that sensory equilibrium is a state which is controlled not by the animal, but by the environmental stimulus.[2] On this basis there would seem to be an infinite number of stationary states corresponding to the infinite number of gradations in the value of I. This is undoubtedly true, because it is possible to adapt *Mya* to any intensity, between complete darkness and the brightest sunlight.

However, it is also true that a certain addition (ΔI) is necessary to a given adaptation intensity, I, before a response of definite magnitude takes place. We know that to produce a given response, the light must produce a constant amount of fresh P and A. Therefore, it should be true that the organism is capable of distinguishing between the two stationary states produced by two intensities only when the transition from one to the other involves a constant increment in the concentration of P and A in the sensory system. It is this proposition which must be tested and borne out by the data we have secured with *Mya*.

VI.

1. Consider the initial adaptation intensity, I, the discrimination increment (or stimulating intensity), ΔI, and the final total intensity, $I + \Delta I$, to which the animal becomes adapted immediately after responding to it. Let x_1 be the concentration of P and A at the stationary state produced by the initial adapting intensity I, so that

$$KI = \frac{x_1^2}{a - x_1}. \tag{5}$$

Also let x_2 be the concentration of P and A at the stationary state resulting from the final intensity, $I + \Delta I$, so that

$$K(I + \Delta I) = \frac{x_2^2}{a - x_2}. \tag{6}$$

[2] *Cf.* Hecht (1922–23, *c*), p. 575 *et seq.*

It is clear that the transition from the stationary state represented by equation (5) to the stationary state given by equation (6) involves two things. First, it involves an increase in concentration of P and A equal to $x_2 - x_1$. Second, it involves a response of a definite magnitude on the part of the animal. Since a given response always requires

TABLE III.*

Photochemical Decomposition Produced in Transition from Stationary State Caused by Initial Adapting Intensity to Stationary State Caused by Final Intensity. The Latter Is Composed of Initial Intensity Plus Added Stimulating Intensity. $K = 0.1$

Initial intensity. I	Conc. of $P + A$. x_1	Final intensity. $I + \Delta I$	Conc. of $P + A$. x_2	Increase in conc. $x_2 - x_1$
m.c.	*per cent*	*m.c.*	*per cent*	*per cent*
0.574	2.4	194.7	35.4	33.0
2.38	4.8	309.4	42.3	37.5
8.57	8.8	428.6	47.5	38.7
35.4	17.1	647.4	54.3	37.2
73.7	23.7	1,158.	64.3	40.6
412.5	46.8	2,768.	78.0	31.2
1,100.	63.4	11,330.	92.5	29.1
Average.....				35.3

* The change from complete dark adaptation ($I = 0$) to the stimulating intensity ($I + \Delta I = 29.9$) is not included here. In this case $x_2 - x_1 = 15.9$, a value obviously much less than those found when the initial condition is a light adaptation stationary state. This phenomenon, which is at present obscure, has already appeared under similar conditions previously (*cf.* Hecht, 1922–23, *c*, Tables III and IV).

the same amount of freshly formed P and A it follows that the values of $x_2 - x_1$ must be the same no matter what the experimental values of I and ΔI happen to be.

In Table III are given the results of the calculations for the first set of data presented in Table II. It is clear that the calculations support strikingly the requirement that $x_2 - x_1$ be constant. The values of $x_2 - x_1$ vary to a certain extent among themselves in what may be a regular way, so that perhaps a small additional factor may be involved in their determination. But considering that the adaptation intensity varies between 0.5 and 1,100 m.c. and the stimulating intensity

between 200 and 10,000 m.c., a variation of about 10 per cent is of little consequence to the fundamental conclusion of constancy.[3]

2. It may be worth while to point out that computation of the other sets of data given in Table II shows essentially the same agreement as given in Table III. This is of course to be expected from the similarities in the behavior of the five sets of data as shown in Fig. 3, since they are all derived from the same series of experiments. Because the magnitudes of ΔI are progressively less as the chosen reaction time increases, the value of $x_2 - x_1$ for each set is correspondingly smaller. Similarly the value of the constant K changes somewhat from one series to another, though the reason for this is not quite clear.

However, the salient and significant point in these calculations is that they prove the deductions from the original assumptions of the photosensory mechanism in *Mya*. For this animal to show the same degree of discrimination between two intensities, I and $I + \Delta I$, there must be decomposed in the sensory system $S \rightleftharpoons P + A$ a constant amount of S. This constant amount is produced by the light, regardless of the way in which the required amount of added light, ΔI, varies in relation to the original amount of light, I.

VII.

1. Having established this, it becomes clear not only why the ratio $\frac{\Delta I}{I}$ cannot be constant as required by the Weber-Fechner law, but why

[3] The equation of the stationary state as derived in this paper depends on the assumption that the photochemical system absorbs light weakly. It is apparent that this is an approximation which will hold over only a moderate range of intensities, and consequently a variation in $x_2 - x_1$ is to be expected on this ground. Certain it is that an analysis of similar data in terms of the assumption of strong absorption (which is mathematically the same as if the concentration of S is so large as not to change noticeably) does not accord with the experimental facts (Hecht (1922–23, *c*), p. 563 *et seq.*). It is probable that an assumption of moderate absorption would account for the data better than one of weak absorption. However, since the variation in $x_2 - x_1$ is comparatively small, I have avoided presenting the more complex mathematical considerations involved in the assumption of moderate absorption. Eventually such an analysis may have to be made, especially in the study of the sensitivity of the eye, in which the intensity range is very great.

it must vary as it does. Consider

$$KI = \frac{x^2}{a - x}$$

the equation of the stationary state of the reaction $S \rightleftharpoons P + A$. Assuming any value of K, one can compute x for a series of intensities, I. Using $K = 0.1$ as we have found it in these experiments and in previous ones with *Mya*,[4] I have plotted in Fig. 6 the relation between log I and x, and between log I and $(a-x)$. K, being a constant, has

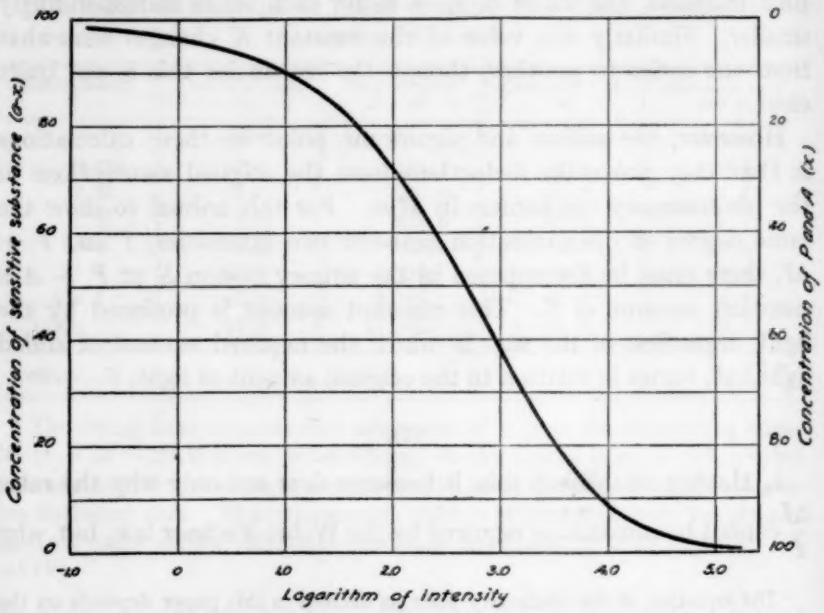

FIG. 6. Concentration of S and $P + A$ at the stationary state produced at any intensity. Note that between $x = 20$, and $x = 90$, the photochemical effect is practically proportional to the logarithm of the intensity.

no influence on the nature of the equation, and hence none on the shape of the curve; it controls the units in which I is given and therefore merely determines the position of the curve on the axis of abscissæ.

Take any point on the curve. It will correspond to a stationary state at a given value of I. To produce a response corresponding to a certain intensity discrimination, the concentration of $P + A$, x_1, will have to move vertically downward a constant distance to a con-

[4] Hecht (1922–23, *c*), p. 570.

centration x_2. Note the behavior of log ΔI, as the value of x progresses downward by regular increments equal to $x_2 - x_1$. Log ΔI is at first large, then it decreases regularly until it reaches a minimum at about $x = 60$ per cent concentration of P and A, when it again begins to increase steadily. Below $x = 60$, log ΔI steadily decreases as log I increases, whereas after that point log ΔI increases also, but always faster than log I. Beginning at the top of the curve, the difference between log ΔI and log I will at first decrease, and after about $x = 60$ will begin to increase. The ratio $\frac{\Delta I}{I}$ is determined by the value log ΔI − log I; therefore, this ratio cannot be constant for *Mya*, but must decrease with increasing initial intensity, and after passing through a minimum, must increase with increasing intensity.

For purposes of approximation the central part of the curve in Fig. 6, say between 20 and 90 per cent concentration of P and A, may without great error be considered a straight line. In this range, then, the concentration of P and A produced by the light is proportional to the logarithm of the intensity, an experimental verification of which already exists (Hecht 1919–20, *b*). Even if the central part be treated as a straight line, it also follows that $\frac{\Delta I}{I}$ cannot possibly be constant. The value log I increases steadily, whereas log ΔI is constant. Therefore, log ΔI − log I or log $\frac{\Delta I}{I}$ must steadily decrease in value until the straight line approximation no longer holds and $\frac{\Delta I}{I}$ begins to increase. In any event the fact cannot be gainsaid that the ratio $\frac{\Delta I}{I}$ not only cannot be constant as demanded by Weber's law, but for *Mya* at least must vary in a definite manner, as indeed it does.

2. An interesting point is apparent from equation (4) of the stationary state. What is the intensity required for the complete decomposition of S? In this case $a - x = 0$, which then gives that $I = \infty$. This means that not only can *Mya* become adapted to any intensity of light, but that even at the highest intensities it will still retain a certain degree of sensitivity. Both these consequences are in conformity with the known experimental facts.

The quantitative behavior of *Mya* with regard to light as demonstrated under the significant conditions treated in this paper, therefore, follows logically from the properties of the photochemical system which we have assumed to represent its sense organ for the reception of light. In addition the analysis of the experiments has laid a basis for the investigation of the nature of intensity discrimination in more complicated systems like the eye.

SUMMARY.

1. A method of experimentation is described which enables one to record objectively and quantitatively the discrimination by *Mya* between two intensities of illumination to which it is successively exposed. The indicator for this discrimination is a response at a given reaction time.

2. From the data so obtained it is found that the difference, ΔI, between the two intensities bears no constant relation to the initial intensity, I. Instead, the ratio $\frac{\Delta I}{I}$ varies in a consistent manner with I. As the latter increases, the ratio decreases to a certain point, after which it increases.

3. The data are analyzed in terms of the photochemical mechanism previously proposed for the sensitivity of *Mya* to light. It is shown that for the animal to discriminate by means of a given reaction between one intensity and another, the transition from one to the other must be accompanied by the decomposition of a constant amount of photosensitive substance in the sense organ.

4. A mathematical treatment of the behavior of the photochemical mechanism shows not only that the ratio $\frac{\Delta I}{I}$ cannot be constant as required by the Weber-Fechner law, but that it must vary in the way in which it does. The behavior of *Mya* under these conditions, therefore, supports the validity of the hypothetical physicochemical mechanism suggested for its sensitivity.

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A MATHEMATICAL TREATMENT OF THE ELECTRICAL CONDUCTIVITY OF COLLOIDS AND CELL SUSPENSIONS.

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Many extensive investigations have been made for the purpose of determining the electrical conductivity of cell suspensions such as blood, body tissues, bacterial suspensions, and the like; the biological importance of this constant being that it would give some idea of the permeability of the cells (or the cell walls) in question. The investigations of the conductivity of blood by Bugarszky and Tangl,[1] Fraenckel,[2] Oker-Blom,[3] Roth,[4] Stewart,[5] and others are well known. These investigators found that the red corpuscles act as perfect insulators,[6] so that the electric current exclusively passes in the space between them. Oker-Blom, for instance, showed that the resistance of a blood cell suspension of a certain volume concentra-

[1] Bugarszky, S., and Tangl, F., Eine Methode zur Bestimmung des relativen Volums der Blutkörperchen und des Plasmas, *Zentr. Physiol.*, 1897–98, xi, 297.

[2] Fraenckel, P., Ueber die Bestimmung des Blutkörperchenvolumens aus der elektrischen Leitfähigkeit, *Z. klin. Med.*, 1904, lii, 470.

[3] Oker-Blom, M., Thierische Säfte und Gewebe in physikalisch-chemischer Beziehung, *Arch. ges. Physiol.*, 1900, lxxix, 510.

[4] Roth, W., Elektrische Leitfähigkeit thierischer Flüssigkeiten, *Zentr. Physiol.*, 1897–98, xi, 271.

[5] Stewart, G. N., The behaviour of the hemoglobin and electrolytes of the coloured corpuscles when blood is laked. *J. Physiol.*, 1899, xxiv, 211. The relative volume of weight of corpuscles and plasma in blood, 356. The mechanism of hemolysis with special reference to the relations of electrolytes to cells, *J. Pharmacol. and Exp. Therap.*, 1909–10, i, 49.

[6] It is to be understood that this apparently high resistance to an electric current of low frequency may be wholly due to polarization at the surfaces of the red corpuscles.

tion was equal to the resistance of a suspension of quartz sand of equal volume concentration in the same suspending medium. The ratio of the resistance of the blood suspension to the resistance of the suspending medium in this case is, therefore, a function of the volume concentration of the corpuscles alone. Experience shows that this function is independent of the size and only slightly dependent on the shape of the suspended particles. In the case of suspensions of cells such as bacteria, part of the current generally goes through the intercellular liquid and part goes through the cells. The resulting conductivity of the suspension is, therefore, a function both of the specific conductivity of the suspending medium and of the specific conductivity of the cellular substance as well as of the volume concentration.

The purpose of the present paper is to derive a formula whereby the specific conductivity of the cellular substance may be calculated from observed values of the specific conductivity of suspensions of the cells in question and of the suspending medium. This formula will be here derived for the simple case of a dilute suspension of spherical homogeneous cells. Nevertheless, the formula can be applied also with fair accuracy to the more general case of a suspension of homogeneous ellipsoids, for it is found that up to a quite large value for the eccentricity of the ellipsoids the same formula as that for homogeneous spheres is applicable. This is in accord with the findings of Oker-Blom and Fraenckel mentioned above, that the conductivity of suspensions of red corpuscles and of quartz sand depends only on the volume concentration of the suspensions.

It may be well at this point to consider briefly the assumption that the cells are homogeneous. In general, a single cell comprises many regions of varying conductivities, as indicated, for instance, by the very large polarization capacity of all living cells. Thus, it is probable that all cells are surrounded by a thin membrane which is semipermeable and in consequence has a comparatively high resistance. However, the formula applicable to a cell consisting of several concentric layers of different conductivities will be identical with that which we shall develop for a homogeneous sphere, the specific conductivity of the homogeneous sphere being replaced by a

certain average value of the conductivities of the different layers of the non-homogeneous cell.[7]

We shall, therefore, consider the case of a suspension of homogeneous spheres (radius a, specific conductivity of cell material k_1) in a medium of specific conductivity k_2. We shall assume that the suspension is so diluted that each sphere acts as if it were alone in the suspending medium—that is, that the spheres are so far apart

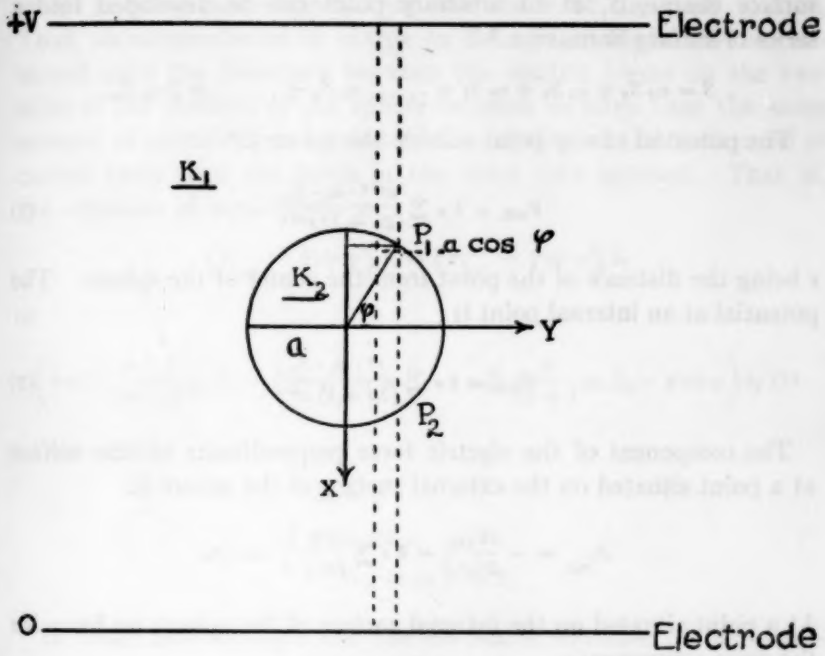

FIG. 1.

that the current lines become parallel in the space between them. As will be shown below, a comparison with experimental data shows that within an accuracy of a few per cent this assumption is fulfilled with concentrations from zero up to 30 per cent.

[7] The treatment of the general case of an ellipsoid having a surface layer of a conductivity different from that of its interior will be taken up in a paper which will appear in the *Physical Review*. This case includes that of a homogeneous polarizable ellipsoid.

Let us consider first the case of a single sphere suspended in the medium (Fig. 1). We assume that the medium is placed in an electrolytic cell with a volume of 1 cc. and that a constant electrical current, i, passes through the suspension. The difference in potential of the electrodes we shall designate as V. Since the electrodes are 1 cm. apart, V is equal to the electric force. The surface of the sphere is the seat of a certain distribution of surface charges. The surface charge, S, at an arbitrary point can be developed into a series of surface harmonics.[8]

$$S = \alpha_0 S_0 + \alpha_1 S_1 + \alpha_2 S_2 + \ldots\ldots \alpha_n S_n + \ldots\ldots = \Sigma\, \alpha_n S_n.$$

The potential at any point outside the sphere is[9]

$$V_{\text{ext.}} = 4\pi \Sigma \frac{a^{n+2} \cdot \alpha_n \cdot S_n}{(2n+1)\, r^{n+1}} \tag{1}$$

r being the distance of the point from the center of the sphere. The potential at an internal point is

$$V_{\text{int.}} = 4\pi \Sigma \frac{r^n \cdot \alpha_n \cdot S_n}{(2n+1)\, a^{n-1}} \tag{2}$$

The component of the electric force perpendicular to the surface at a point situated on the external surface of the sphere is

$$F_{r_{\text{ext.}}} = -\frac{dV_{\text{ext.}}}{dr(r=a)} = 4\pi \Sigma \frac{n+1}{2n+1} \cdot \alpha_n \cdot S_n$$

At a point situated on the internal surface of the sphere we have for the same component:

$$F_{r_{\text{int.}}} = -\frac{dV_{\text{int.}}}{dr(r=a)} = -4\pi \Sigma \frac{n}{2n+1}\, \alpha_n \cdot S_n.$$

These forces are the forces due to the electric charges on the spheres. The total forces are obtained by adding the component of the original electric force due to the surface charges on the electrodes of the electrolytic cell. This component is equal to $-V \sin \varphi$ (Fig. 1).

[8] See Jeans, J. H., The mathematical theory of electricity and magnetism, Cambridge, 1920, 206.

The electric force has a discontinuity at the surface of the sphere. At the moment when the current through the suspension is started, the electric force is the same on the two opposite sides of the surface of the sphere. As soon as the current is started, since the conductivity of the substance of the sphere is different from the conductivity of the suspending medium, a different amount of electricity will be carried to the outside of an element of surface from that which is being carried away from the inside at the same instant. Thus, an accumulation of charge on the surface begins and is continued until the difference between the electric forces on the two sides of the surfaces of the sphere becomes so large that the same amount of electricity is carried to the outside of the surface as is carried away from the inside in the same time interval. That is, the equation of equilibrium is

$$\left(F_{r_{\text{ext.}}} - V\sin\varphi\right) k_1 = \left(F_{r_{\text{int.}}} - V\sin\varphi\right) k_2$$

or

$$\left(4\pi\Sigma\,\frac{n+1}{2n+1}\,\alpha_n S_n - V\sin\varphi\right) k_1 = \left(-4\pi\Sigma\,\frac{n}{2n+1}\,\alpha_n S_n - V\sin\varphi\right) k_2 \quad (3)$$

We have[9]

$$S_0 = 1$$
$$S_1 = \sin\varphi$$
$$S_2 = \tfrac{1}{2}\,(3\sin\varphi^2 - 1)$$

In order that the right and the left side of equation (3) be identities it is necessary that α_n be made zero for all values of n except 1. Consequently equation (3) reduces to

$$\left(4\pi\cdot\tfrac{1}{3}\,\alpha_1\sin\varphi - V\sin\varphi\right) k_1 = \left(-4\pi\cdot\tfrac{1}{3}\,\alpha_1\sin\varphi - V\sin\varphi\right) k_2$$

from which we derive the value of α_1.

$$\alpha_1 = \frac{V\,(k_1 - k_2)}{\frac{4\pi}{3}\,(2k_1 + k_2)}$$

[9] Jeans,[8] p. 224.

Substituting this value for α_1 in equation (1) and (2) for the potentials $V_{ext.}$ and $V_{int.}$ we now obtain

$$V_{\substack{ext. \\ r=a}}(\varphi) = a \cdot \frac{V(k_1 - k_2)}{(2k_1 + k_2)} \sin\varphi$$

$$V_{\substack{int. \\ r=a}}(\varphi) = a \cdot \frac{V(k_1 - k_2)}{(2k_1 + k_2)} \sin\varphi$$

The conductivity of the suspension can now be calculated by means of the following method. The space between the electrodes of the electrolytic cell is divided into an infinite number of volume elements, dS, dx; dS being a surface element parallel to the electrodes and dx a line element perpendicular to the electrodes. If F_x is the component of the electric force along x then by using Ohm's law

$$\int\int F_x \cdot dS \cdot dx \cdot k_1 + \int\int F_x \cdot dS \cdot dx \cdot k_2 = \int i dx = V \cdot k \qquad (4)$$

k being the conductivity of the suspension. The first integration is taken over all volume elements outside the sphere, the second integration over all elements inside the sphere.

In carrying through this integration on the left side we integrate all elements situated in the cylinder defined by a fixed dS. If this cylinder does not cross the sphere, its elements contribute solely to the first integral. This contribution is

$$\text{Contr.}_1 = \int\int F_x \, dS \cdot dx \, k_1 = \int k_1 \, dS \cdot \int F_x dx = k_1 V \cdot \int dS = k_1 V \cdot (1 - \pi a^2).$$

The contribution of the cylinders crossing the sphere (as indicated in Fig. 1) to the left side of our equation is

$$\text{Contr.}_2 = \int\int F_x dS \, dx \, k_1 + \int\int F_x dS \, dx k_2 = \int (V - \Delta V) \, dS \, k_1 + \int \Delta V \, dS \, k_2$$

ΔV being the difference in potential between the two points, P_1 and P_2, where the cylinder crosses the surface of the sphere. Then, according to what was found above

$$\Delta V = \underset{r=a}{V_{\text{int.}}}(\varphi) - \underset{r=a}{V_{\text{int.}}}(-\varphi) + 2Va\sin\varphi$$

$$= 2 \cdot V\frac{(k_1 - k_2)}{2k_1 + k_2} a\sin\varphi + 2Va\sin\varphi$$

$$= 2Va \cdot \frac{3k_1}{2k_1 + k_2}\sin\varphi.$$

Consequently taking

$$dS = 2\pi a\cos\varphi\, d(a\cos\varphi)$$

$$= 2\pi a^2\cos\varphi\, d(\cos\varphi)$$

$$\text{Contr.}_2 = \int V\, dS \cdot k_1 + \int_{\frac{\pi}{2}}^{0} \Delta V\,(k_2 - k_1)\, 2\pi a^2\cos\varphi\, d(\cos\varphi)$$

$$= \pi a^2 V k_1 + \int_{\frac{\pi}{2}}^{0} 2Va \cdot \frac{3k_1}{2k_1 + k_2}\sin\varphi\,(k_2 - k_1)\, 2\pi a^2\cos\varphi\, d(\cos\varphi).$$

$$= \pi a^2 V k_1 + \frac{12\pi k_1(k_2 - k_1)}{2k_1 + k_2} Va^3 \int_{\varphi=0}^{\varphi=\frac{\pi}{2}} \sin^2\varphi\, d(\sin\varphi)$$

$$= \pi a^2 V k_1\left(1 + \frac{4(k_2 - k_1)}{2k_1 + k_2} a\right).$$

Using the expressions obtained for Contr.$_1$ and Contr.$_2$ equation (4) becomes

$$V(1 - \pi a^2) k_1 + \pi a^2 V k_1\left(1 + \frac{4(k_2 - k_1)}{2k_1 + k_2} a\right) = Vk$$

$$Vk_1 + \frac{4(k_2 - k_1)}{2k_1 + k_2}\pi a^3 Vk_1 = Vk$$

$$k = k_1 + \frac{4(k_2 - k_1)}{2k_1 + k_2}\pi a^3 k_1.$$

Calling the volume of the sphere ρ we obtain

$$k = \left(1 + \frac{3(k_2 - k_1)}{2k_1 - k_2}\rho\right) k_1$$

or

$$\frac{k}{k_1} = 1 + \frac{3\left(1 - \frac{k_1}{k_2}\right)}{1 + 2\frac{k_1}{k_2}} \cdot \rho \qquad (5)$$

This formula with ρ being the total volume of spheres per cc. will hold for a suspension of spheres so diluted that each sphere deforms the current lines of the original current independently of every other.

We see that $\frac{k}{k_1}$ is a linear function of ρ.

From equation (5) we derive the value for $\frac{k_2}{k_1}$

$$\frac{k_2}{k_1} = \frac{1 + 2\frac{\frac{k}{k_1} - 1}{3\rho}}{1 - \frac{\frac{k}{k_1} - 1}{3\rho}} \tag{6}$$

The above formula shows that for diluted cell suspensions $\frac{\frac{k}{k_1} - 1}{3\rho}$ is independent of the concentration. In Fig. 2 $\frac{k_2}{k_1}$ is plotted against $\frac{\frac{k}{k_1} - 1}{3\rho}$ for values of $\frac{k}{k_1} > 1$, while $\frac{k_1}{k_2}$ is plotted for $\frac{k}{k_1} < 1$.

A test of the theory here developed has been made by applying formula (6) to red corpuscles. As was stated above, red corpuscles are very nearly non-conductors; that is, $k_2 = 0$. According to our theory $\frac{\frac{k}{k_1} - 1}{3\rho}$ should therefore have the constant value of $-\frac{1}{2}$. In Table I are given the values of $\frac{\frac{k}{k_1} - 1}{3\rho}$ calculated from data given by Fraenckel for red corpuscles of man, dog, horse, and cow. It is seen that the experimental value of $\frac{\frac{k}{k_1} - 1}{3\rho}$ has the theoretical value

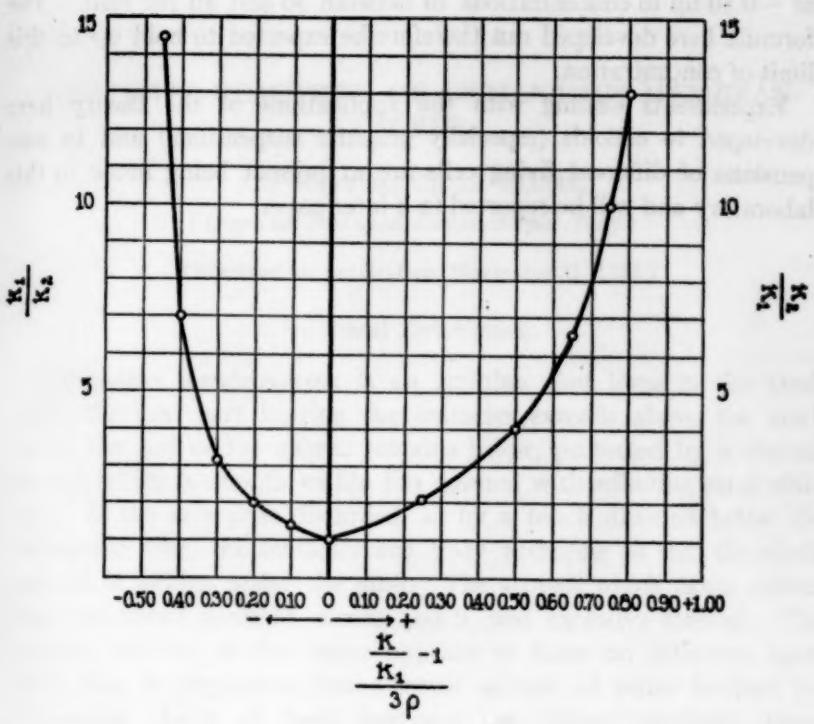

FIG. 2.

TABLE I.

$\frac{\frac{k}{k_1}-1}{3}$	ρ
	per cent
−0.53	10
−0.52	20
−0.50	30
−0.47	40
−0.44	50

of -0.50 up to concentrations of between 30 and 40 per cent. The formula here developed can therefore be expected to hold up to this limit of concentration.

Experiments dealing with the applications of the theory here developed to colloids (especially graphite suspensions) and to suspensions of different living cells are at present being made in this laboratory and will be reported in a later paper.

TROPISTIC REACTIONS OF CERIANTHUS MEMBRANACEUS.

By MARY MITCHELL MOORE.

(*From the Zoological Station, Naples, Italy.*)

(Received for publication, November 21, 1923.)

General Description.

Cerianthus membranaceus is an actinian that lives in the sand. Only the oral part bearing the tentacles extends above the sand, while the rest of the animal remains below, protected by a mucoid sheath which is smooth within but covered with adhering sand without. If the animal is disturbed, as by a touch directed below the tentacular ring, the tentacles and body withdraw so that the whole animal is hidden under the sand. The stimuli which cause retraction are direct sunlight, strong touch, and excessive feeding. The oxygen content of the water appears to have no influence upon retraction or expansion, but a small current of water hastens reexpansion. Lack of food, darkness (or diffuse daylight) favor expansion also. At the aboral end is a small pore, and the end of the outer protecting sheath is always open, possibly allowing water currents to pass through the animal. Loeb[1] describes accurately the formation of a new sheath by mucous secretion after the animal has been removed from the sand and carefully slipped out of its sheath. The whole surface of the body, except tentacles and a small area near the oral disk, actively secretes mucus in which sand particles are soon caught. When the animal is first removed from the sand, the longitudinal (inside layer) muscles contract, the tentacles become limp, and the body hard so that its appearance is that of a short stubby brush. This contraction forces the water out of the body cavity, consequently when the animal relaxes directly after this it has lost all turgor and resembles an empty sac. If, however, it is

[1] Loeb, J., Studies in general physiology, Chicago, 1905, 164.

left undisturbed, it fills with water, regains turgor, and soon burrows into the sand and spreads out its tentacles as before. The animals used in our experiments varied between 3 and 15 cm. in length.

Stereotropism.

Loeb[2] made the observation that *Cerianthus* would dig its way under small blocks of lead placed upon the sand. If instead one places a glass tube upon the glass bottom of the aquarium so that the foot of the animal (which has been removed from its sheath) just touches the open end of the tube, the animal is forced by its positive stereotropism to crawl into the tube, and spread out its tentacles normally. If the tube is too small the animal will enter only part way. If the tube is larger than the animal, then it expands its diameter and shortens its length so that the whole lateral surface of its body is touching the inner surface of the tube. I found that as long as they were fed regularly these animals remained indefinitely long in the tubes open at both ends.

Geotropism.

The glass tube containing *Cerianthus* lies horizontally on the floor of the aquarium. The oral disk bearing its rows of tentacles extends nearly vertically upward, while the foot touches the lower surface of the tube. This condition exists also in the dark, so that we may speak of the oral end as being negatively, the foot, positively geotropic. Specimens of *Cerianthus* in glass tubes retain this position even when placed on the sand, unless the aboral end be tipped down slightly, when the animal burrows into the sand, leaving its glass house behind. If a *Cerianthus* in a glass tube be suspended midway in the water so that no part of the animal touches the aquarium, the oral end extends upward, and the foot touches the lower surface of the tube or hangs down a very little beyond the tube. If, however, the tube is not long enough to support the foot, then both ends of the animal hang down, and tentacles are limp (Fig. 1). Such

[2] Loeb,[1] p. 158.

an animal will regain turgor only when the whole surface of the body is touching some solid object.[3]

Stereotropism in *Cerianthus* may be counterbalanced by the reaction to gravity. For instance, a specimen will crawl upward into a tube placed at 35° with the horizontal, although instead of the usual ½ hour it takes 4 to 5 hours. In such a position the animal will expand its tentacles and eat. But if a *Cerianthus* be suspended oral end down at an angle of 45°, the two tropisms are nearly balanced. One specimen so suspended remained expanded in this position 1 day, but on the 2nd day the foot reacted to gravity by turning back on the body and boring downward inside the tube (Fig. 2). The

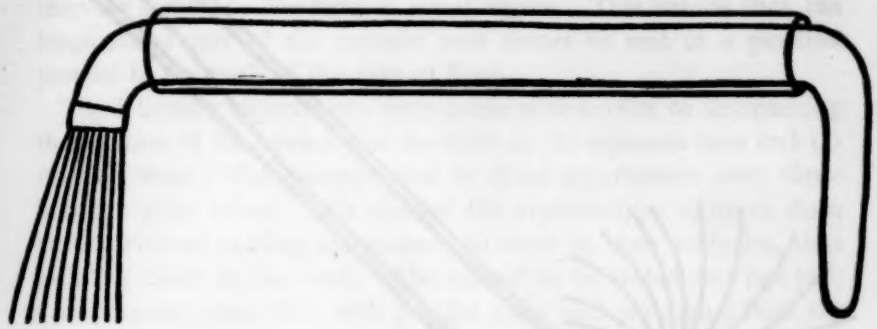

FIG. 1. *Cerianthus* in which only the middle part of the body is in contact with a surface; head and foot hang freely in the water of the aquarium. Note complete loss of turgor.

oral end extended horizontally and tentacles remained expanded for 2 days, during this time taking food as usual. On the 3rd day the oral end and tentacles were withdrawn into the tube, and in 1¾ hours the position of the animal was reversed and normal orientation with respect to gravity was regained. The movement was slow and

[3] When specimens are suspended in tubes the function of the foot in boring holes may be observed. The tip of the foot follows the inside surface of the glass tube in a clockwise direction, in such a way that the body becomes twisted like a corkscrew. At intervals the longitudinal muscles of the body contract, so that if the animal were boring in the sand this would have the effect of drawing the oral end farther under the sand. But the suspended animals only remain hanging in the glass tubes. If the foot is cut off the *Cerianthus* is no longer able to dig a hole but remains lying on the sand in a more or less limp condition.

continuous, apparently being accomplished by ciliary action. Therefore, the critical angle for stereotropism in *Cerianthus* is about 45°.

Heliotropism.

Haime[4] observed that the direct rays of the sun cause complete retraction of *Cerianthus* and that these animals emerge in sunlight but to a slight extent. In summer, in the port of Mahon, he says that

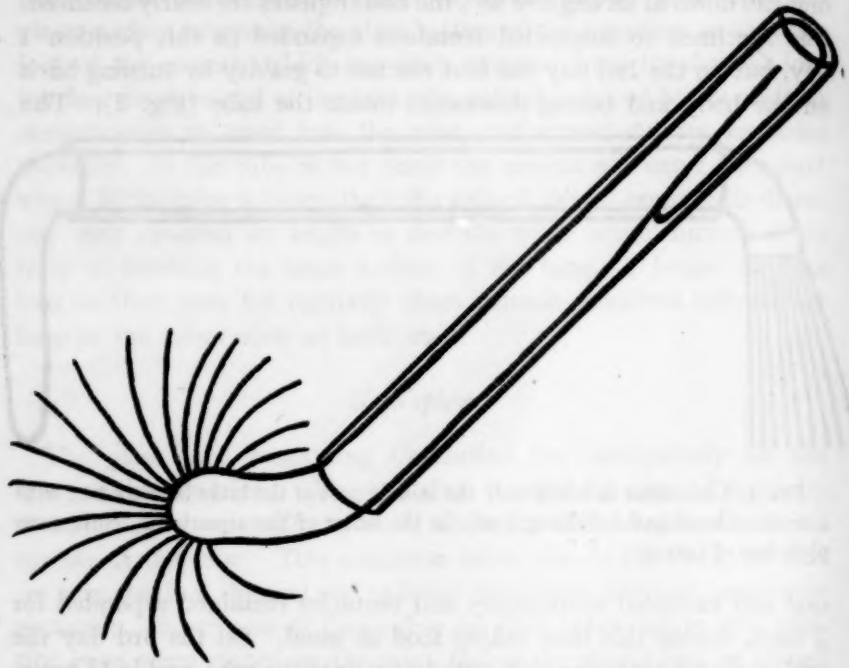

FIG. 2. Tube containing *Cerianthus* suspended at an angle of 45°, showing beginning of movement of the foot downward in response to gravity.

Cerianthus remains under the sand until the sun goes down. He put specimens in tubes of cloth for observation and also studied their embryology, but reports that he did not discover any nervous system.

Nagel[5] (1894) states that if *Cerianthus* has been kept in the dark and is brought into bright daylight the oral disk retracts. C.

[4] Haime, J., *Ann. sc. nat. zool.*, 1854, i, series 4, 341.
[5] Nagel, W. A., *Arch. ges. Physiol.*, 1894, lvii, 495.

Hess[6] in 1913 published photographs showing how *Cerianthus* turns toward the light of an electric bulb, but he did no quantitative experiments.

Light has the general effect of increasing tone. Only in the dark do the animals reach a stage of complete expansion of body and tentacles, the length of the body protruding from the tube or burrow being sometimes as much as three times the length attained in ordinary daylight. When the animal is extended in the dark room it is very sensitive to the light. The oral disk turns toward the electric light in less than a minute, the final orientation with a strong light being such that symmetrical tentacles are equally illuminated, since they are struck by the light at equal angles. This means that the longitudinal axis of the exposed part comes to rest in a position parallel to the path of the rays of light.

The following experiments were made with a view to determining the relation of the intensity of the light to (1) exposure time and (2) reaction time. The animals used in these experiments were those living in glass tubes. This enabled the experimenter to move them about without causing the animals to draw in their tentacles, thus avoiding delay in the work. The animal to be tested was put into a rectangular glass dish with parallel sides and containing fresh sea water. The dish was placed on a table in the dark room, and an hour allowed for necessary dark adaptation to take place in the animal. Between exposures the animal was kept in the dark for ½ hour. The electric bulb was enclosed in a black box with a rectangular slit covered with a black paper shutter which could be raised or lowered. The time was measured with a stop-watch. The intensity of the light was varied by changing the distance of the light from the animal.

Measurements were made of the amount of light necessary to cause the heliotropic reaction (exposure time). The animals were illuminated for a measured length of time with a light of known intensity, and under a red glower it was observed whether the animal turned the oral disk toward the light. If the amount of light was sufficient to cause turning, the time of exposure was lessened until by successive trials an exposure was found which was just sufficient to cause the animal to turn.

[6] Hess, C., *Zool. Jahrb., Abt. allg. Zool. u. Physiol. Tiere*, 1913, xxxiii, 387.

After the exposure time a few seconds elapse before the animal turns which may be called the latent period. The total reaction time was considered to be the time which elapsed between the instant when the beam of light was thrown on the animal, and the instant when the ends of tentacles began to move. The total reaction time is, therefore, the sum of the exposure time and the latent period. The experiments were repeated on three animals and averages of at least six readings at each intensity are given.

Table I shows that in *Cerianthus* the relation between the exposure time and the intensity of the light may be expressed mathematically by the equation $K = I \times T$, in which I = the intensity of the light, T = the exposure time, and K is a constant. This equation expresses the Bunsen-Roscoe law.

TABLE I.

Relation between Intensity of Light, and the Time of Exposure Necessary to Produce Heliotropic Turning.

Relative intensity.*	Average presentation time (obs.).	Presentation time (calc. $K = 0.46$).	$I \times T$.
	sec.	*sec.*	
1.00	0.8	0.4	0.8
0.25	2.6	1.8	0.65
0.11	4.1	4.1	0.45
0.062	7.6	7.4	0.47
0.04	11.5	11.5	0.46
0.027	(No turning.)		

*8900 candle meters were taken as unity.

The first two observed values of K are large, possibly because of the difficulty of accurately measuring small amounts of time with the crude apparatus at hand. Since, however, the last three values show such good agreement, the constant was taken equal to their average value. 1/100 actual intensity is plotted against observed exposure time in Fig. 3, Curve A.

Total reaction time is plotted against 1/100 actual intensity in Curve B. The theoretical curve is of the same type as that in Curve A, namely a hyperbola of the type $XY = K$. If the point 10,0 is taken as the origin, and intensities are taken as 1/100 their actual value in meter candles, then the equation is $I \times (t + 0.25) = 28.29$. Abscissæ show actual reaction time, and ordinates 1/100 intensities.

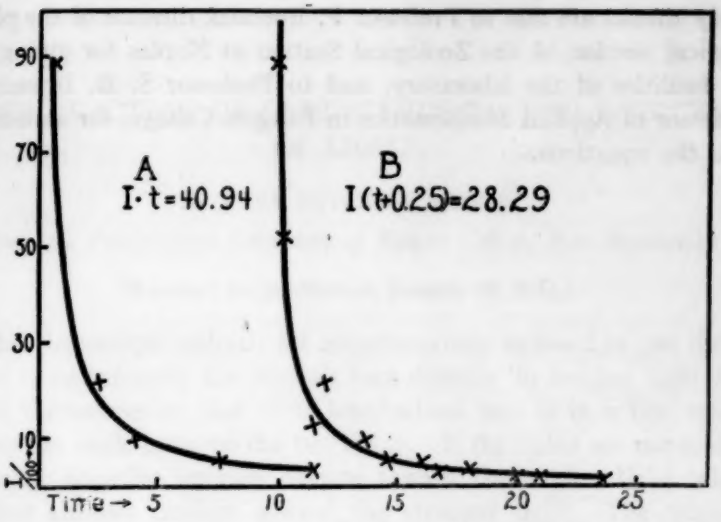

FIG. 3. Curve *A* shows the relation of exposure time to intensity. Intensity 89 is taken as unity, so that the constant = (0.46 × 89) = 40.94. The curve follows the equation $I \times t = 40.94$. Curve *B* shows the relation between reaction time and intensity. The curve follows the equation $I \times (t + 0.25) = 28.29$, if the point 10,0 is taken as the origin, and 1/100 actual values of intensities are used.

CONCLUSIONS.

1. *Cerianthus* shows stereotropism and if fed regularly will remain indefinitely in glass tubes. The animal retains turgor only so long as the entire surface of the body is in contact with the glass.

2. *Cerianthus* is positively geotropic as regards the foot and body. It will work upward into a glass tube suspended at an angle of 35° as a result of stereotropism, but if the tube is raised to an angle of 45° the foot turns back and the animal reverses its position in the tube so that foot is down, head, up. Thus at an angle of 45° stereotropism and geotropism very nearly balance each other.

3. Light increases the muscle tone of *Cerianthus;* this results in positive phototropism. $I \times t = K$ where I is the intensity of the light, t is exposure time, and K, a constant, = 0.46. Where reaction time is considered, $I\ (t + k) = K$, in which I is intensity of light, t is reaction time, $k = 0.25$, and $K = 28.29$. The two equations prove the operation of the Bunsen-Roscoe law.

My thanks are due to Professor F. Bottazzi, director of the physiological section of the Zoological Station at Naples for giving me the facilities of the laboratory, and to Professor S. E. Brasefield, Professor of Applied Mathematics in Rutgers College, for assistance with the equations.

THE REACTION OF CERIANTHUS TO TWO SOURCES OF LIGHT.

By MARY MITCHELL MOORE.

(From the Physiological Laboratory of Rutgers College, New Brunswick.)

(Received for publication, January 19, 1924.)

When heliotropic animals are simultaneously exposed to two lights of the same intensity the animals turn directly to neither light but orient themselves so that their longitudinal axis is in a line which bisects the angle between the two lights. If the lights are unequal in intensity, negative animals deviate toward the weaker light, while positive animals deviate toward the stronger light. The tropism theory demands that when the animal comes to rest, the effective illumination on the two sides of the animal shall be equal, since the muscles on each side are then contracted to the same extent.[1] When the lights are unequal in intensity the physical intensity of the lights may be equalized when the photosensitive areas of the animal are inclined toward the lights at certain angles. The angle at which equal illumination occurs, with two lights of a given difference in intensity, varies with the angle at which the two beams of light cross each other. For instance, equal illumination is possible for positive animals when the beams cross each other at 90°, while at 180° equal illumination is not possible as long as the animal deviates toward the stronger of the two lights. The following summary may help to make this relationship clear.

Conditions under Which Physical Illumination on Two Sides of the Animal is Equal or Unequal.

1. Lights shining at 90°.—(*A*) When the lights are equal, equal illumination is possible only at 45°, (whether the photosensory surfaces are parallel or non-parallel, the animal positive or negative).

[1] Loeb, J., Forced movements, tropisms, and animal conduct, Monographs on experimental biology, Philadelphia and London, 1918.

(*B*) When the lights are unequal, equal illumination is possible only at certain angles physically determined by the ratio of the intensities (whether the photosensory surfaces are parallel or not, the animal positive or negative).

(*C*) Whether the lights are equal or unequal, the illumination is unequal at all other angles.

2. Lights shining at 180°.—(*A*) Equal illumination is possible:

(*a*) At all angles when the lights are equal, the photosensory surfaces are parallel, the animal positive or negative.

(*b*) At certain angles when the lights are unequal, the photosensory surfaces not parallel, and the animal is negative.

(*B*) Unequal illumination occurs:

(*a*) At all angles when the lights are unequal, the photosensory surfaces are parallel, the animal positive or negative.

(*b*) At all angles when the lights are unequal, the photosensory surfaces are not parallel, and the animal is positive.

The orientation of animals when subjected to two lights of varying intensities has been studied only in those cases in which there might occur equal illumination on the two sides of the animal. The angle of orientation for positive *Balanus* larvæ has been determined by Loeb and Northrop,[2] the two unequal lights shining at 90°. The same authors[3] have measured the angle of orientation for negative *Limulus*, the lights again at 90°. Crozier[4] found that in holothurians whose photosensitive areas are parallel, no orientation could be obtained when equal lights are opposed at 180°. When one light is much stronger than the other the animals turn away from the stronger light. Patten[5] measured the angle of orientation for negative blow-fly larvæ, the lights of varying intensities being reflected to shine at 180° across the larvæ. In all these cases the animals might be considered to have taken such a position that the effective illumination was equal on their two sides. The case for the positive animal which orients when the two lights are opposed at 180° has not up to this time been considered.

[2] Loeb, J., and Northrop, J. H., *Proc. Nat. Acad. Sc.*, 1917, iii, 539.
[3] Northrop, J. H., and Loeb, J., *J. Gen. Physiol.*, 1922–23, v, 581.
[4] Crozier, W. J., *Am. J. Physiol.*, 1917, xliii, 510.
[5] Patten, B. M., *J. Exp. Zool.*, 1914, xvii, 213.

It therefore seemed of interest to study the positions assumed by a positive animal such as *Cerianthus*, since it is evident that equal external illumination on two sides of the animal is impossible as long as the lights are unequal and the animal is positive. As has been stated in a former paper,[6] if this animal is illuminated from one side only, it turns the oral end toward the light. The angle turned which in this case I have not yet determined quantitatively, depends in some way upon the intensity of the illumination. At weak intensities if no turning occurs within about 20 seconds the animal never reacts to the light at all. If after an interval in the dark the animal is again tested with a light of stronger intensity, the head turns part way. Only with relatively strong lights did the head region turn through the whole 90° toward the light. In the experiments with two lights, these effective intensities were used, and not the weaker lights. If *Cerianthus* is allowed to remain in the dark the oral end tends to lie in the same straight line as the tail. This is then the normal position of the body of the animal. If, however, two lights of equal intensity shine across the animal, the oral end takes up such a position that it lies perpendicular to the line connecting the two lights. This position is held, whether the tail of the animal be bent toward the left or right, and it may be called the neutral position. When the lights are turned on, the immediate effect upon the animal is an increase in the muscle tone, so that the oral disk is not so far extended from the tube as in the dark. Three animals, one much larger than the other two, and all living in glass tubes, were used to find the relation between the angle turned and the ratio of the intensities of the two lights.

The experiment is as follows (Fig. 1): The animal is placed in a rectangular glass dish *OM* filled with fresh sea water. On the table under the dish is put a piece of white paper with a line *PQ* ruled on it in pencil. The animal is placed so that its longitudinal axis is parallel to this line, and then allowed to remain in the dark room for 1 hour. The two lights, *A* and *B*, are arranged on opposite sides of the dish so that their beams fall in a straight line across the animal's end. The intensity of one light (*A*) is kept constant at 6,800 meter

[6] Moore, M. M., *J. Gen. Physiol.*, 1923–24, vi, 385.

candles, while the other (B) is varied by increasing its distance from the animal so that the ratio is successively equal to 1, 2, 4, and α, that is, extinction of the weaker light. After the lights are turned on, the animal moves from the neutral position to another position

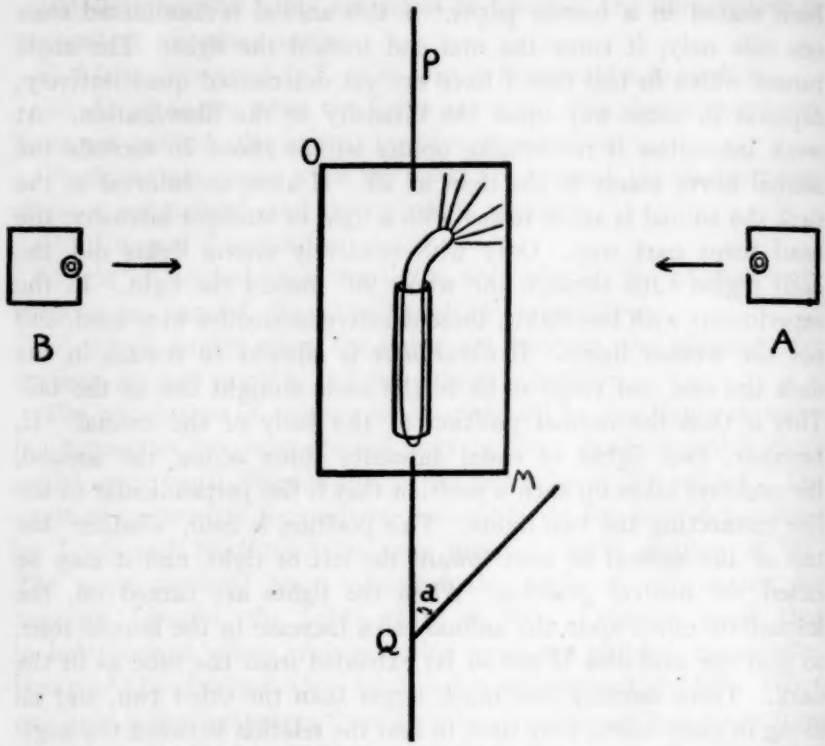

FIG. 1. Position assumed by *Cerianthus* when acted upon by two lights of unequal intensity, the rays of light falling on the animal at right angles to the longitudinal axis of the body. OM is the aquarium, PQ the longitudinal axis of the body, A and B are lights in which the intensity of A is four times the intensity of B. a is the angle which the head of *Cerianthus* at equilibrium makes with the axis QP.

which it maintains as long as the lights shine upon it in the same ratio. Now while the animal is in this position another line, QM, is drawn parallel to the side of the anterior end of the animal, *i.e.*, the longitudinal axis of the head. The angle turned is found by projecting this line to the axis PQ to intersect at Q. The individual

variation on different days was very slight occasioning an error not greater than that involved in determining the angle. Between readings 1 hour was allowed for dark adaptation.

The experiments showed that if the two lights are of equal intensity, the oral disk is extended at right angles to the line connecting the lights. But if one light is twice as strong as the other, the oral disk moves approximately 27° toward the stronger light, while if one light is four times as strong as the other, the angle is approximately 43°. If only one light is used and that one of sufficient intensity, the animal turns through the whole 90° toward the light. This

TABLE I.

Experiments on Cerianthus Showing the Relation between the Angle Turned and the Ratio between the Intensities of Two Lights.

Ratio.	$\text{Log}_{10}\frac{I_2}{I_1}$.	a (observed).	a (calculated, $K = 0.615$).	Tan a.	K.
1:1	0	0°	0°	0	
2:1	0.3	27°	26°	0.5095	0.59
4:1	0.6	43°	44°	0.9325	0.64
4:0	α	90°	90°	α	

relation may be expressed mathematically by the following equation

$$\log_{10}\frac{I_2}{I_1} = K \tan a,$$

if $\frac{I_2}{I_1}$ is the ratio between the two lights, a is the angle turned from the neutral position, and K is a constant. It is obvious that the tangent of the angle is directly proportional to the logarithm of the ratio between the two lights (Table I).

This equation not only expresses the relation between the angle turned from the neutral position and the relative intensities of the two lights in the case of *Cerianthus*, but also expresses this relation as shown by the data published by Patten for blow-fly larvæ, by Loeb and Northrop for *Balanus* larvæ, and by Northrop and Loeb for *Limulus* (Tables II, III, and IV). Cole[7] has shown that when

[7] Cole, W. H., *J. Gen. Physiol.*, 1922–23, v, 417.

Limulus is acted upon by one light the logarithmic relation holds namely, $E = K \log I$, in which E is the photochemical effect, I is intensity, and K is a constant. Assuming that the effect on each side of the *Limulus* is proportional to the log I on that side, [8,9] then

$E_1 = K_1 \cdot \log I_1$ on one side and

$E_2 = K_2 \cdot \log I_2$ on the other.

Then,

$$\frac{E_1}{K_1} = \log I_1$$

$$\frac{E_2}{K_2} = \log I_2$$

Subtracting,

$$\frac{E_2}{K_2} - \frac{E_1}{K_1} = \log \frac{I_2}{I_1}$$

$$\frac{E_2K_1 - E_1K_2}{K_1K_2} = \log \frac{I_2}{I_1}$$

From the experiments of Northrop and Loeb, it can be shown (Table II) that in *Limulus* the ratio $\frac{E_2K_1 - E_1K_2}{K_1 K_2}$ is equal to K tan a, a being the angle turned from the neutral position. It may be pointed out that in all the cases where the angles of orientation have been measured when animals were acted upon by two sources of light, the animals did not orient themselves at angles such that equal physical illumination occurred on the two sides. Northrop and Loeb supposed that in *Limulus* the photosensory surfaces are

[8] Hartline, H. K., *J. Gen. Physiol.*, 1923–24, vi, 137.

[9] Since this paper was presented for publication Hartline[8] has published some data on the reactions of certain isopods to one source of light which support this assumption. He also has derived an equation, $R = k \cdot t \cdot \log \frac{I_2}{I_1}$ in which R is the "tendency to turn." As the author states, this equation holds for equal effective intensities. However, the left-hand member, R, if measured by the number of degrees turned and divided by $\log \frac{I_2}{I_1}$ does not give a constant. It therefore appears that the accurate expression for the "tendency to turn" is "tan a."

TABLE II.

Calculation Showing That the Orientation of Limulus When Illuminated by Two Lights Follows the Same Rule as Cerianthus.

Ratio: $\frac{I_2}{I_1}$.	Log $\frac{I_2}{I_1}$.	Observed angle.	α = angle turned from neutral position.	Tan α.	$\frac{\text{Log } \frac{I_2}{I_1}}{\tan \alpha} = K$.
1:1	0	45.5°	0°	0	
2:1	0.3	60.2°	14.7°	0.2617	1.14
4:1	0.6	67.3°	21.8°	0.4006	1.49
4:0	α	89.4°	43.9°	0.9629	
Average					1.31

Northrop and Loeb, *Limulus.*

TABLE III.

Calculation Showing That Balanus Larvæ Follow the Same Rule as Cerianthus.

Ratio: $\frac{I_2}{I_1}$.	Log $\frac{I_2}{I_1}$.	Observed angle.	α = angle turned from neutral position.	Tan α.	$\frac{\text{Log } \frac{I_2}{I_1}}{\tan \alpha} = K$.
1:1	0	45.6°	0°	0	
2:1	0.3	40°	5.6°	0.096	3.12
4:1	0.6	34.4°	11.2°	0.197	3.04
10:1	1.0	28.8°	16.8°	0.302	3.31
Average					3.15

Loeb and Northrop, *Balanus* larvæ.

TABLE IV.

Calculation Showing That the Same Rule Applies to the Negative Blow-Fly Larvæ.

Percentage difference between the two lights.	Ratio: $\frac{I_2}{I_1}$.	Log $\frac{I_2}{I_1}$.	α = average angular deflection toward weaker light.	Tan α.	$\frac{\text{Log } \frac{I_2}{I_1}}{\tan \alpha} = K$
0	12:12 = 1.0	0.0	0.09°	0.001	(Indeterminate.)
8⅓	12:11 = 1.09	0.0374	2.77°	0.048	0.779
16⅔	12:10 = 1.20	0.0792	5.75°	0.102	0.776
25	12:9 = 1.33	0.124	8.86°	0.155	0.800
33⅓	12:8 = 1.50	0.176	11.92°	0.209	0.842
50	12:6 = 2.0	0.301	20.28°	0.369	0.815
66⅔	12:4 = 3.0	0.477	30.90°	0.596	0.800
83⅓	12:2 = 6.0	0.778	46.81°	1.066	0.729
100	12:0 = (indeterminate).		77.56°	4.51	
Average					0.790

Patten, blow-fly larvæ.

placed at such angles as to make the effective illumination the same on the two eyes of the animal although they were unable to calculate what these angles may be. Patten calculated the angle at which the photosensory elements in the blow-fly larvæ would have to be inclined, if the animals took such a position that the illumination on the two sides were equal. However, it has not been determined whether the photosensory areas in the blow-fly larvæ are actually so inclined. It therefore appears that in all these experiments on heliotropic turning of animals illuminated by two lights, the apparent agreement of the results with the tropism theory rests upon an assumption which may or may not be valid.

The theory has assumed that the amount of photochemically changed substance is the same on the two sides of the animal when a position of equilibrium has been reached. Even if the effective physical illumination is not the same on the two sides, as is the case in these experiments with *Cerianthus*, yet possibly the photochemical effect inside the animal may be equalized in some way not determined. The intensity outside the animal is greatly modified by sense organs so that we only know that the photochemical effect inside the animal varies with the light outside. That equal amounts of light may not have the same effect in opposite sides of the same animal was demonstrated by Patten who found blow-fly larvæ which were unsymmetrical with respect to their photosensitive areas. These animals when illuminated equally on their two sides turned toward the side of less sensitivity, since they were negative. Garrey[10] also found that it is possible to produce differential sensitivity in the two eyes of the robber-fly by removing the covering from one eye after it has been covered for 2 or 3 days. Such an animal circles toward the more sensitive side.

Another possible explanation of the seeming contradiction to the basic assumption of the theory of phototropism is that here we may have not a final orientation such as may be the case with free moving animals, but a tropistic bending comparable to the experiments of Garrey[10] on insects in which unequal illumination in the two eyes was produced by blacking one eye. In these animals forced positions

[10] Garrey, W. E., *Proc. Nat. Acad. Sc.*, 1917, iii, 602.

were assumed as a result of unequal illumination. The same forced unilateral tension occurs when certain animals are subjected to a constant electric current.[11–13]

In the experiments on *Cerianthus* the two sides of the animal were unequally illuminated, and like Garrey's flies, the muscle tone on the two sides of the animal was correspondingly unequal. But on the other hand it has been shown that not only in sessile animals like *Cerianthus* but also in moving animals the same relation holds between the intensity of the two lights, and the angle turned from the neutral position where the physical illumination is the same. May we not, therefore, conclude that in all cases of heliotropic bending in animals differences in illumination cause corresponding differences in muscle tonus which in the free moving animal tend to bring the animal into a position where equal illumination occurs? The equation is a mathematical expression of the fact that the muscle tonus on a given side is proportional to the logarithm of the intensity of illumination on that side. Therefore, since the angle turned is the result of muscle tonus, the tangent is proportional to the logarithm of the ratio between the two intensities.[14]

CONCLUSIONS.

1. In case *Cerianthus* is acted upon by two lights of unequal intensities the relation between the angle turned and the ratio between the intensities of the two lights is mathematically expressed by the equation $\log_{10} \frac{I_2}{I_1} = K \tan a$, in which $\frac{I_2}{I_1}$ is the ratio of the two lights, a is the angle turned from the neutral position, and $K = 0.615$.

2. According to published data from experiments on other animals, the relation between the angle turned and the ratio between the intensities of the two lights may be expressed by the same equation.

I wish to thank Professor S. E. Brasefield, of Rutgers College, for helpful advice.

[11] Moore, A. R., and Kellogg, F. M., *Biol. Bull.*, 1916, xxx, 131.
[12] Loeb, J., and Maxwell, S. S., *Arch. ges. Physiol.*, 1896, lxiii, 121.
[13] Loeb, J., and Garrey, W. E., *Arch. ges. Physiol.*, 1896–97, lxv, 41.
[14] Moore, M. M., *Proc. Soc. Exp. Biol. and Med.*, 1923, xxi, 54.

LUMINESCENCE IN MNEMIOPSIS.*

By A. R. MOORE.

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(Received for publication, January 18, 1924.)

The Ctenophore, *Mnemiopsis leidyi*, is a southward ranging form in New Jersey waters, occurring annually in greater or less numbers in Barnegat Bay.[1] The animal is provided with eight rows of paddle plates (Fig. 1) and underlying each row of plates is a

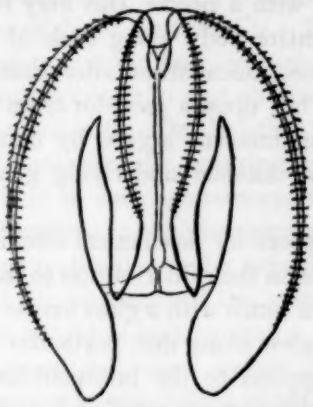

FIG. 1. Drawing of *Mnemiopsis*. The rows of paddle plates show where luminescence appears when a dark adapted animal is stimulated.

* The preliminary experiments of this paper were made in May, 1919, in the house boat of Dr. T. C .Nelson, then stationed at Tuckerton, New Jersey. During that season *Mnemiopsis* was very abundant in the waters of Barnegat Bay, but only rarely were specimens found in the 3 succeeding years. During the summer and fall of 1923, however, *Mnemiopsis* occurred again in great numbers (T. C. Nelson, Report of New Jersey Experiment Station, 1923). It thus became possible to finish some of the work begun in 1919. I am very glad to take this occasion to thank Dr. Nelson for giving me the use of his house boat laboratory and for much assistance in securing material both in 1919 and during the past season.

[1] Nelson, T. C., *Anat. Rec.*, 1923, xxvi, 381.

corresponding row of luminescent organs. When the dark adapted animal is stimulated mechanically the luminescent organs respond with a glow of blue-green light, so that in the dark the outline of the individual is defined by eight meridians of light. This reaction is inhibited completely if the animal has been exposed to sunlight for a few minutes. In the dark, after a time, the power of luminescence is recovered, and Peters[2] found that the recovery process is facilitated by mechanical agitation.

The Physiology of Luminescence.

It can be shown that the luminescence reaction is normally under the control of the nervous system. If a dark adapted animal be touched at one point with a needle, this may result in luminescence spreading over the entire body along each of the eight meridians. Hence the action of mechanical stimulation cannot be directly on the luminescent material but upon a receptor from which the impulse is transmitted to the luminescent organs by conducting paths. This corresponds to the mechanism underlying general luminescence in *Pelagia*.[3]

Moreover, the receptors for mechanical stimulation of the luminescent organs are limited in their distribution to the eight rows of paddle plates, because, while a touch with a glass needle applied to a meridian causes an immediate glow along that particular row of paddle plates, similar stimulation applied to the intermeridian does not result in luminescence unless the pressure applied is sufficient to cause some deformation of the meridian. On the other hand, contact stimulation *at any point* whatever causes cessation of movement of the swimming plates and closure of the oral lobes. It is therefore clear that while the swimming plates and muscles are innervated by the general nerve-net system, the luminescent organs are limited in their innervation to the receptor cells and conducting tissue of the eight meridians. The experiment also proves that luminescence does not depend upon excitation of contractile tissue.

[2] Peters, A. W., *J. Exp. Zool.*, 1905, ii, 103.

[3] Heymans, C., and Moore, A. R., *Compt. rend. Soc. biol.*, 1923, lxxxix, 430. Heymans, C., and Moore, A. R., *J. Gen. Physiol.*, 1923–24, vi, 273.

The luminescent material may be studied apart from the animal by means of luminescent paper obtained in the same way as a similar indicator paper was prepared from the medusa *Pelagia noctiluca*.[3] This was done by rolling the animal over a piece of filter paper. It is necessary to be sure there are no large pieces of tissue left on the paper since these contain intact cells which are stimulated by NaCl. Such indicator paper does not glow in the dark until rubbed, or wet with an appropriate solution. Solutions of pure salts isosmotic with the sea water of Barnegat Bay, namely of concentration M/4, show characteristic action on the indicator paper. All solutions were brought to pH 7.7. It was further found what variations in pH between pH 6 and 8 were without effect on the reaction. The indicator glows in solutions of K_2SO_4, KCl, $CaCl_2$, $SrCl_2$, $MgSO_4$ but does not glow in solutions of NaCl and $MgCl_2$. That $CaCl_2$ and $SrCl_2$ should act in a stimulatory sense and NaCl should not is worthy of note since this is a result opposite to that obtained in the case of nerve and muscle with these two salts. On the other hand if entire animals are immersed in NaCl solution the resulting hyperirritability of the tissues causes the animal to emit spontaneous flashes of light. This experiment adds confirmation to the view expressed above that in the animal the luminescent material is under nervous control.

While the power of luminescence of the animal is suppressed in a very few minutes with a light of high intensity, the luminescent properties of the luminescent substance are not destroyed by the same treatment. An individual in which light of 681 c. p. suppressed luminescence in 8 minutes, yielded luminescent material which showed no deterioration after 30 minutes exposure to the same light. This is shown by the fact that the paper glows when rubbed or torn or put into KCl solution. However, exposure for a few minutes to direct sunlight or artificial illumination long continued does destroy the luminescent properties of the indicator paper. These facts suggest that in the suppression of luminescence *in the animal* the light acts not directly on the luminescent substance itself, but indirectly through a photoreceptor cell and nervous connections. The situation is analogous to that of mechanical stimulation. Weak mechanical stimulation of the animal causes the luminescent reaction, but vigorous rubbing is necessary to cause the luminescent material to glow.

Similarly, luminescence in the animal is suppressed by a quantity of light which is not sufficient to affect the luminescent material on filter paper.

The character of the photoreceptor connections can be shown by illuminating one part of a large animal. This results in suppression of luminescence only in the area of the animal which has been subject to illumination. Thus if the aboral half has been illuminated with sufficient quantity of light, mechanical stimulation of a meridian anywhere results in luminescence of the oral half alone. Hence there can be no extensive linear connection between the photoreceptors and the luminescent cells.

An unusual relation of luminescence to temperature was discovered. If animals living at a temperature, of 20°C. be cooled to 9° no luminescence is at once elicitable. This was noted by Peters. But if the cooling is continued to 3°C. of course no luminescence is obtainable although the paddle plates continue beating.[4] (This shows that the motion of the plates and luminescence are independent variables.) If now the temperature is raised to 7°C. luminescence appears upon stimulation. Furthermore, if the animals are kept a few hours at 3°C. luminescence occurs regularly on stimulation at that temperature.

Inhibition of Luminescence by Light.

It was next attempted to determine the relation between the intensity of illumination and the time required for the suppression of luminescence. The procedure was as follows: Each animal was put into a beaker containing sea water to a depth of 1 cm. and then dark adapted for 30 minutes. The experiment was made by exposing the dark adapted animals in groups of three to a light of known intensity until no luminescence was observable when the light was extinguished and the dish rotated. Observations were always made with dark adapted eyes. The point of extinction was determined by testing the animal every 2 minutes when such time had elapsed that it was judged the exposure was very nearly sufficient. It was always possible to approximate this time because the intensity of the luminescence obtainable decreases with continued exposure to illumination. The time required

[4] I have seen the paddle plates beating at a temperature of −0.6°C.

for suppression was taken as the mean between the time of the last reading at which luminescence appeared and the next reading which showed complete suppression. After this point had been found the animal was again kept in darkness 30 minutes at the end of which time its power of luminescence was completely restored and another determination could be made. Each individual showed consistent readings but there were considerable differences between individuals in the time necessary for the light at a given intensity to suppress luminescence (Table I). The fact that each individual yields consistent results for the five intensities is further proof that the luminescent material reacts in a closed system. Animals which had been freshly brought into the

TABLE I.

Intensity.	Time.						Average time.
	A	B	C	D	E	F	
meter candles	*min.*	*min.*	*min.*	*min.*	*min.*	*min.*	*min.*
170	23	17	16	17	17	19	18.2
305	17	13	11	11	18	12	13.7
436	8	6	6	8	11	7	7.7
681	7	3	5	2	12	4	5.5
1,210	5	1	6	2	7	2	3.8

A, B, C, D, E, F, denote the six individuals used in this experiment.

laboratory required a longer time for suppression of luminescence with light of a given intensity than those which had been kept in the laboratory several days. As a source of light a tungsten lamp of 109 c.p. was used. The lamp was clamped to a tall iron stand and five different intensities were obtained by fixing the light at distances of 80, 60, 50, 40, and 30 cm. which yielded intensities of 170, 305, 436, 681, 1,210 m.c., respectively. The temperature was 20–21°C.

When the suppression time in minutes for each intensity had been determined for a given series of animals the average time for each intensity was calculated. These average values in turn form the basis for the mean values shown in the plot (Fig. 2, Table II). With freshly collected animals it was impracticable to obtain readings for the lowest intensity of light since the exposure time was too long, but with animals which had been kept in dishes in the laboratory for

several days all the exposure times were shorter, and such animals were always used for the lower intensities. On the other hand, for the high intensities only fresh animals were employed in order that the exposure times might be as long as possible, thus increasing the accu-

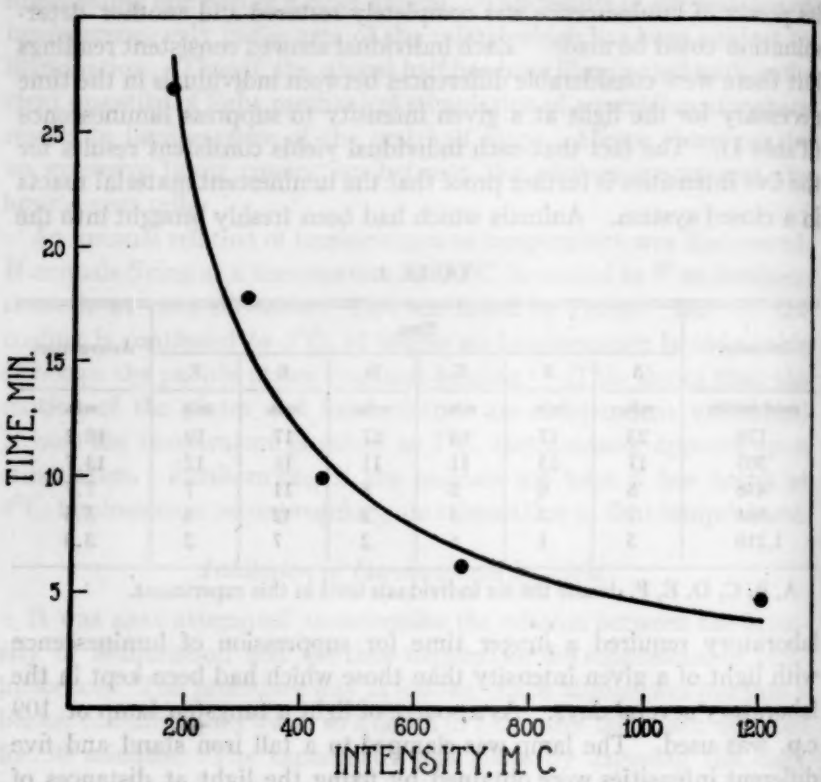

FIG. 2. Abscissæ represent intensities of light (I) and ordinates the average time (t) in minutes required at the intensity given to suppress luminescence. In the equation $I \times t = K$, $K = 4{,}776$, average value, calculated values of t are obtained for each intensity by substituting this value of K and the values of the intensities in the equation. From the calculated values of t the curve is drawn; circles indicate average observed values of t for corresponding intensities of light.

racy of the measurements. It turned out that while the curves yielded by the different experiments were parallel, those obtained from the animals kept in the laboratory for a day or more were always displaced toward the x-axis. In order to construct the curve (Fig. 2), it was

necessary to reduce all sets of measurements to the same standard of value. This was done by setting the time at intensity 436 m.c. in each series as 10 minutes and by simple proportion calculating all the other exposure times of the series. The method used in calculating the mean of the averages may be illustrated by the following example. There were three series of experiments made with 305 m.c. intensity, two involved six animals, and one, nine animals. The average exposure time for the first series (six animals) was 17.8 minutes; for the second (nine animals), 19.2 minutes; and for the third (six animals), 15.8 minutes.

Hence,

$6 \times 17.8 = 106.8$

$9 \times 19.2 = 172.8$

$6 \times 15.8 = 94.8$

Total animals 21) 374.4

17.8 mean exposure time at 305 m.c.

TABLE II.

Intensity.	No. of animals.	Time.	K
meter candles		*min.*	
170	12	26.9	4,573
305	21	17.8	5,429
436	30	10.0	4,360
681	35	6.2	4,222
1,210	20	4.8	5,808
Average			4,776

In similar fashion the mean exposure time for each of the five intensities was calculated. The results are summarized in Table II. Each exposure time multiplied by the corresponding intensity yields a value for K which is fairly constant for the series and gives an average value for K of 4,776. Since the product of intensity and time is a constant, given the same end-point, it follows that the Bunsen-Roscoe law holds for the effect of light on the luminescence reaction in *Mnemiopsis*. This relation proves the photochemical nature of the action of light in this form.

Theoretical Considerations.

The foregoing experiments were made with a view to getting an insight into the physiology of the luminescent reaction in *Mnemiopsis*.[5] While the picture is at present by no means complete it may be of some value to present a view of the process which the experiments up to this time seem to support. In order to account for the results of illumination in suppressing luminescence it is necessary to suppose that the photoreceptor cells are connected with luminescent cells in the simplest possible fashion, in which there are neither linear nor lateral branches. The assumption that light acts on photoreceptor cells and not on luminescent material in suppressing luminescence in the animal is based on the fact that the luminescence reaction is more delicately responsive to the action of light in the animal than in the indicator paper. Since, then, the light acts on the animal only locally, in suppressing luminescence, the photoreceptors cannot have appreciable lateral nervous connections. As to the effects of mechanical stimulation, tactile receptors for luminescence occur only along the lines of the paddle plates and are connected with a conducting system only along this line; there are no intermeridianal branches. In contrast to this limited conducting arrangement for the luminescence reaction, the tactile receptors for muscular and ciliary action possess, through the nerve net, two or three dimensional connections with all parts of the organism.

If we consider the reaction system of the luminescent material as it acts in the animal we may provisionally adopt the following scheme.

$$D \underset{\text{dark}}{\overset{2\ \text{illumination}}{\leftrightarrows}} A \underset{\text{rest}}{\overset{1\ \text{excitation}}{\rightleftarrows}} L$$

It is assumed that the system is a closed one. The evidence indicates that the reactions are simultaneous and of the first class, namely side reactions,[6] in which A is the luminescent substance in the resting,

[5] Moore, A. R., *Proc. Soc. Exp. Biol. and Med.*, 1923, xxi, 52.

[6] Lewis, W. C. McC., A system of physical chemistry, London and New York, 1920, i, 398.

dark adapted animal, L is the light giving form of the substance, formation of which is catalyzed by stimulation, D is a decomposition product, formation of which is facilitated by illumination of sufficient intensity and duration. Such a scheme makes intelligible the experiment of Peters[2] in which he found that stimulation hastens the recovery of luminescence in an animal which had previously been light adapted, since excitation removes A from the system as rapidly as it is formed and thus facilitates the "dark" reaction. Likewise the "rest" reaction goes on even under strong illumination. It is evident that D cannot be directly converted into L, and it also seems probable that L cannot be changed into D without passing through the intermediary stage A.

If we assume that both reactions involving the decomposition of A are set up by nervous impulses, then each luminescent organ must receive two types of innervation. A nerve impulse arriving from a tactile receptor sets going reaction 1 and luminescence results, but an impulse coming from the light receptor causes A to decompose (reaction 2) into an altogether different substance, D, which cannot give rise to luminescence until it has been reformed into substance A. The luminescent substance thus receives double innervation and the character of the decomposition is determined by the type of nerve fiber stimulated.

CONCLUSIONS.

1. In the dark adapted *Mnemiopsis*, mechanical stimulation causes luminescence along the eight rows of paddle plates. The tactile receptors for this reaction lie only in the paddle plate rows, and are connected only longitudinally along these rows.

2. The tactile receptors for ciliary and muscular movement are distributed generally over the surface and are connected by a nerve net.

3. Luminescence may occur at 3°C. provided the animal has been kept sufficiently long at that temperature. Ciliary action goes on at − 0.6°C.

4. Luminescent paper made by spreading the luminescent secretion of *Mnemiopsis* on filter paper, yields the following effects. The paper shows luminescence in solutions of K_2SO_4, KCl, $MgSO_4$, $SrCl_2$,

$CaCl_2$; no luminescence in NaCl, $MgCl_2$. Changes in pH value of salt solutions between pH 6 and 8 do not affect the phenomenon. Illumination of the paper with strong light for longer time than necessary to suppress luminescence in the living animal has no effect on the subsequent luminescence of the paper. Hence in the animal, light affects luminescence through the photoreceptor system; the nervous system carries the impulse to the luminescent organs.

5. The power of luminescence of the animal is suppressed by sufficiently intense light, the relation between the intensity and the time requisite being expressed by the equation for the Bunsen-Roscoe photochemical law, namely, $I \cdot t = K$.

6. It is suggested that the reaction scheme involved in luminescence is of the following form

$$D \underset{\text{dark}}{\overset{2}{\underset{\text{illumination}}{\leftrightarrows}}} A \underset{\text{rest}}{\overset{1}{\underset{\text{excitation}}{\rightleftarrows}}} L$$

in which A is the luminescent substance in the resting, dark adapted animal, L is the light-giving decomposition product, and D is a product which does not yield light.

7. The luminescent substance receives double innervation and the character of the decomposition is determined by the type of nerve fiber stimulated.

A CELL FOR THE MEASUREMENT OF CATAPHORESIS OF ULTRAMICROSCOPIC PARTICLES.

By M. KUNITZ.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, November 26, 1923.)

In a recent publication Northrop[1] describes a convenient cell for microscopic cataphoresis measurements which can easily be made by cementing together microscopic slides. The writer has modified the cell so that it can be employed for measurements of cataphoresis of particles of ultramicroscopic size such as colloidal gold, etc. The modification consists in cementing slides in such a manner as to make it possible for a strong beam of light to pass through the front edge of the cell. The diagrams in Fig. 1 show the construction of the cell. The slides are lettered alphabetically in the order in which they are cemented together by means of de Khotinsky cement. Slide *d* is perforated and is made from an ordinary thick slide which has a circular flat depression in the middle. This depression is easily cut away by means of a glass cutter and one edge of the slide is then ground off, leaving a space of 1 mm. between the perforation and the edge.

Construction of the Cell.—Slide *b* is cemented on top of Slide *a*, leaving about 1.5 cm. of Slide *a* exposed. Slide *c* comes on top of *b*, and then Slide *d* on top of Slide *c*, taking care that the ground edge of *d* coincides in the same vertical plane with the exposed edge of Slide *a*. The long front edge of the cell is ground smooth, and Slide *e* is cemented to it. On top of Slide *e* come the blocks *f* and *g*, leaving about 2 cm. of the middle portion of Slide *e* exposed. The ends of the cell are then ground smooth, the inside of the cell is cleaned by means of lens paper and acetone, and end-tubes are then cemented on. The end-tubes consist of two short pieces (4 to 5 cm. long) of thick-walled glass tubing, one end of which is widened and flat-

[1] Northrop, J. H., *J. Gen. Physiol.*, 1921–22, iv, 629.

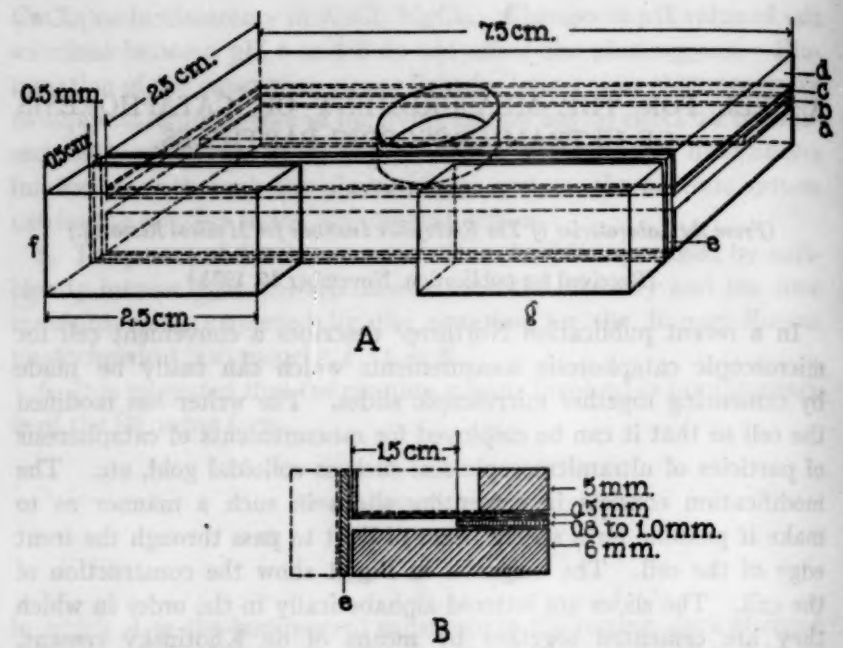

FIG. 1. *A*, perspective view of the cell. *B*, transverse section through the middle of the cell.

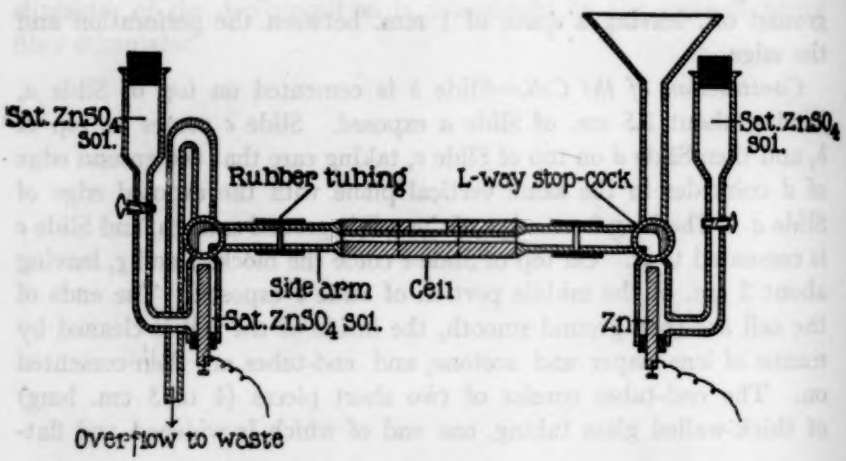

FIG. 2. Longitudinal section through the cataphoresis apparatus.

tened and then ground smooth so as to fit well to the end of the cell. As in the case of Northrop's arrangement, non-polarizable Zn-$ZnSO_4$ electrodes are employed. The electrodes designed by Northrop were slightly modified by adding reservoirs for saturated $ZnSO_4$ solution, thus making it possible to renew readily the $ZnSO_4$ solution around the zinc electrodes. Fig. 2 gives a sectional view of the whole cell assembled, together with the modified zinc electrodes.

Calibration of the Cell.—In employing the apparatus for cataphoresis measurements it is necessary to know the drop in potential per cm. length of the part of the cell where the observations are made, when a known E.M.F. is applied to the terminals of the zinc electrodes. The drop of potential per cm. length of the cell proper can be calculated, with an approximation sufficient for the accuracy of cataphoresis experiments, from the actual measurements of the lengths and the inner cross-sectional areas of all the parts of the apparatus used in the electric circuit, with the exception of the parts containing saturated $ZnSO_4$ solution. The resistance of saturated $ZnSO_4$ solution is very small in comparison with the electrolytes usually employed in cataphoresis measurements; hence the potential drop across the $ZnSO_4$ portions of the circuit can be neglected. The calculation of the drop per cm. length of the cell is based on the principle that the drop per cm. length of a conductor of a non-uniform cross-section is inversely proportional to the cross-sectional area of any part of the conductor. If e_c and e_1 are the drops per cm. length of the cell, whose cross-sectional area is A_c, and of any other part of the apparatus, whose cross-sectional area is A_1, then

$$e_1 = e_c \frac{A_c}{A_1}.$$

Hence

$$\text{the total drop } E = e_c \left(L_c + L_1 \times \frac{A_c}{A_1} + L_2 \times \frac{A_c}{A_2} + \ldots\ldots \right)$$

in which L_c, L_1, L_2, respectively, are the lengths in cm. of the various parts of apparatus. If $L_1 \times \frac{A_c}{A_1}$ and $L_2 \times \frac{A_c}{A_2}$ respectively, are designated as the equivalent lengths of the various parts of the apparatus in terms of the length L_c of the cell proper, then the drop

per unit length of the cell is equal to the total P.D. as measured by a voltmeter connected across the terminals of the zinc electrodes divided by the sum of all the equivalent lengths, or

$$e_c = \frac{E}{L_c + L_1 \times \frac{A_c}{A_1} + L_2 \times \frac{A_c}{A_2} + \ldots\ldots}$$

Another formula for calculating the value of e_c is given by Northrop in his publication mentioned before, where the reader may find more details as to the technique of operating the apparatus as well as to the method employed in determining the actual velocity of electrical migration of suspended particles.

The cell described here has been successfully employed for several months, using a simplified Zsigmondy[2] arrangement for obtaining a strong beam of light, which consists, (1) of a small Leitz arc lamp which is using 4 to 5 amperes and which is provided with a clock mechanism for leading the carbons,[3] (2) of a double-convex lens, and (3) of a microscope objective (Zeiss A) attached to a microscope barrel. This is mounted in a horizontal position on a stand which can be raised or lowered by means of a fine screw. A microscope provided with a Zeiss objective of 16 mm. focal length and a No. 12 compensating ocular, in which a cross-sectional microscale is placed, is used for taking observations.

The cell can also be used for ordinary microscopic cataphoresis measurements by allowing light to reach the bottom of the cell as usual. But the writer has found the ultramicroscopic method to be of advantage even in cases of large microscopic particles, as it is much easier to focus and less tiresome for the eye to make many observations when the ultramicroscope method is employed than using an ordinary microscope method.

[2] Zsigmondy, R., Kolloidchemie, Leipsic, 2nd edition, 1918.

[3] The arc lamp has been recently replaced in this laboratory, at the kind suggestion of Dr. L. Waldo, by a convenient incandescent lamp supplied by the Pathescope Company of America, Aeolian Hall, New York.

THE KINETICS OF TRYPSIN DIGESTION.

II. Conditions under Which the Reaction is Monomolecular.

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A review of the literature on the kinetics of enzyme actions shows that in general the reactions do not obey the simple monomolecular formula. This is particularly true of the proteolytic enzymes. Most of the workers in the field have concluded that the discrepancies are due to some complicating factor, usually considered to be an intermediate compound between enzyme and substrate, and that the reaction is really in agreement with the mass law if these side reactions are taken into account. Henri[1] attempted to formulate an equation that would include these disturbing factors and a number of similar equations have since been proposed. The results in general, however, have been inconclusive owing largely to the fact that the equations inevitably contain several arbitrary constants. The agreement with the experimental results, therefore, loses most of its significance and can always be considered empirical. This was clearly shown by the work of Nelson and Hitchcock[2] on invertase. They found that many of the proposed equations could be reduced to the same form, which, however, did not accurately represent the data even when the constants were derived by the method of least squares. Results such as these have led Bayliss[3] and others to the conclusion that the reactions are not homogeneous at all and that it is useless to attempt to reconcile the results with the mass law. It is possible, however, to attack the problem in another way. Instead of attempting to account for all the complicating factors mathematically, experimental conditions can

[1] Henri, V., Lois générales de l'action des diastases, Paris, 1903, 79.

[2] Nelson, J. M., and Hitchcock, D. I., *J. Am. Chem. Soc.*, 1921, xliii, 2632.

[3] Bayliss, W. M., The nature of enzyme action, New York, 3rd edition, 1914.

be chosen in such a way as to eliminate these complications. The present paper is an attempt to study trypsin digestion under such conditions and as will be seen the reaction agrees very well with the theory.

The kinetics of trypsin digestion as ordinarily carried out were outlined by Bayliss.[4] Bayliss found that the reaction was not monomolecular, and that the amount of substrate hydrolyzed did not increase in proportion as the enzyme concentration increased nor as the substrate concentration increased. In other words, the reaction disagreed with the simple mass action theory in almost every respect and Bayliss concluded that the reaction could not be considered homogeneous, but that the results were due to an intermediate adsorption compound. Bayliss also showed, however, that the enzyme was inactivated during the reaction and was inhibited by the products formed. The writer has shown[5] that both of these reactions by themselves agree with the law of mass action. It is impossible, however, to introduce the necessary corrections into the formula owing to the complexity of the relations involved.

There is no doubt that the reaction takes place between the water, enzyme, and protein, so that, assuming one molecule to react, the equation governing the reaction must have the general form

$$-\frac{dS}{dT} = K \text{ (substrate) (enzyme) (water)}$$

in which S is the substrate concentration, T the time, and the terms in parentheses concentrations. By using dilute solutions, the water concentration may be considered constant and so disappears from the equation. If the enzyme is also considered constant, the ordinary monomolecular formula is obtained. It is easy to show experimentally, however, that the enzyme is not constant, due to two effects; first, it is continually undergoing spontaneous inactivation, the more rapidly the higher the temperature; and second, it is continually being inhibited by combination with the products of hydrolysis. This effect will be less the greater the relative amount of enzyme compared to the products There is another complication due to the fact that

[4] Bayliss, W. M., *Arch. sc. biol.*, 1909, xi, suppl., 261.

[5] Northrop, J. H., *J. Gen. Physiol.*, 1921–22, iv, 245, 261.

the digestion of protein is not one reaction but a series of consecutive reactions and there is every reason to suppose that these reactions occur at different rates. For this reason alone, therefore, the final result would not agree with the monomolecular formula. These complications cannot be corrected for mathematically but it is possible to choose experimental conditions under which they are reduced to a minimum. If the reaction is carried out at low temperatures, the inactivation of the enzyme is negligible. If a large amount of enzyme is used, the inhibiting effect of the products is also very small, and if the first step in the reaction alone is followed, the effect of the consecutive reactions disappears. These conditions can be fulfilled by digesting casein at 0° with a large amount of trypsin and following the reaction by the disappearance of the protein as indicated by its precipitation with trichloroacetic acid.

TABLE I.

Hydrolysis of Casein.

1 per cent casein.				3.0 per cent casein.		
Time.	$A-x$ N/50 protein N per 1 cc.		$KE = \frac{1}{T} \log \frac{A}{A-x}$	Time.	$A-x$ N/50 protein N per 0.5 cc.	$KE = \frac{1}{T} \log \frac{A}{A-x}$
	Observed.	Calculated.				
hrs.	*cc.*	*cc.*		*hrs.*	*cc.*	
0.01	5.68	5.68		0	8.60	
0.11	5.38	5.376	0.235	0.20	7.20	0.39
0.215	5.08	5.075	0.236	0.40	6.02	0.39
0.53	4.25	4.27	0.242	0.80	4.37	0.36
1.0	3.16	3.29	0.255	1.60	2.20	0.37
2.51	1.44	1.444	0.238	3.40	0.57	0.35
4.00	0.62	0.637	0.238			

The result of an experiment carried out under these conditions is shown in Table I. The figures show that the reaction follows the monomolecular time curve with a considerable degree of accuracy. It furnishes very strong proof of the fact that the discrepancies ordinarily observed are due to the complications enumerated above and which have been experimentally eliminated in this case.

Experimental Procedure.

The casein was prepared by precipitation from acid as described by the writer.[6] The trypsin (Fairchild's) was dissolved in water, titrated to pH 4.5, filtered, and brought back to pH 7.4. This removes most of the accompanying protein. It is necessary to use a powerful buffer solution in order to prevent changes in pH. In these experiments a borate buffer was used. It was prepared by suspending 0.25 mol boric acid in water, titrating to the desired pH, and then making up to 1 liter. The casein is then dissolved in this solution and the pH adjusted again with NaOH. Even under these conditions there is a change in pH which disturbs the reaction when concentrated casein is used. 20 cc. of trypsin solution at 0°C. were added to 130 cc. of 1 per cent casein solution in M/4 borate buffer pH 7.6, also at 0°C. 10 cc. samples were taken at intervals into 10 cc. of cold 10 per cent trichloroacetic acid.[7] The solutions were kept overnight in the ice box, centrifuged, and the precipitate dissolved in alkali and made up to 10 cc. 1 or sometimes 2 cc. of this solution was then analyzed for total nitrogen by Folin and Farmer's[8] micro method. The results are expressed as cc. N/50 alkali. The figures are the average of 4 to 6 determinations. This method determines only the first step in the digestion, *i.e.* the hydrolysis of the casein molecule itself.

Effect of Varying the Trypsin Concentration.

If the formula used above is correct, the value of KE should increase directly as the concentration of trypsin, provided no complicating factors are introduced, *i.e.* the trypsin cannot be too dilute or the effect of the products will become noticeable and the constants will decrease. The trypsin solution itself must be free from such products or the free trypsin will not increase as rapidly as the total trypsin concentration. The result of an experiment in which the trypsin was varied is shown below.

Relative trypsin concentration, E	1	2	4	8
Observed velocity constant, KE	0.057	0.11	0.24	0.50
Corrected velocity constant, K	0.057	0.055	0.060	0.062

The formula evidently correctly expresses the effect of varying the trypsin concentration.

[6] Northrop, J. H., *J. Gen. Physiol.*, 1922–23, v, 749.
[7] Hiller, A., and Van Slyke, D. D., *J. Biol. Chem.*, 1922, liii, 253.
[8] Folin, O., and Farmer, C. J., *J. Biol. Chem.*, 1912, xi, 493.

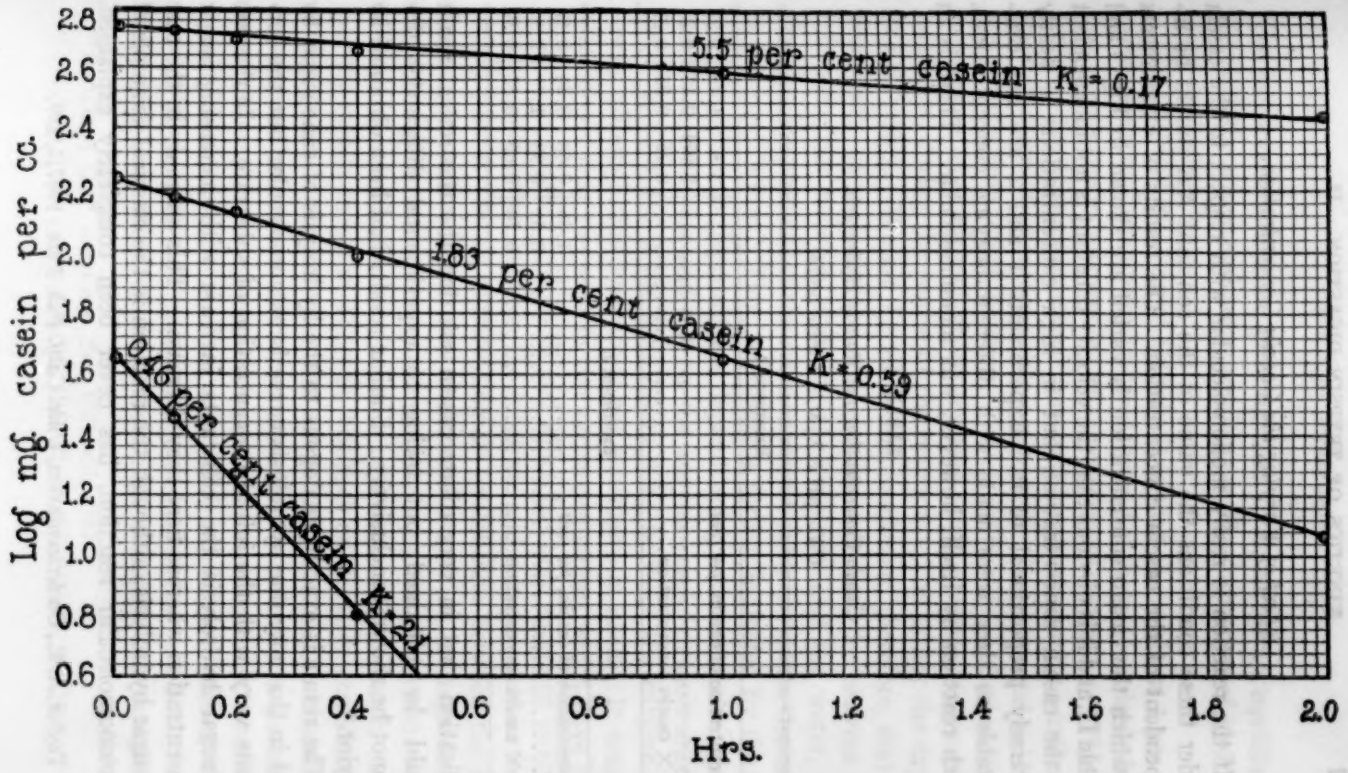

FIG. 1. The rate of hydrolysis of various casein concentrations with constant trypsin.

Effect of Varying the Casein Concentration.

If the reaction is really monomolecular with respect to the casein under these conditions the value of the constant KE should be independent of the casein concentration. The results of experiments in which the casein concentration is varied are shown in Fig. 1 and Table II, and it is obvious that the velocity constant is not independent of the casein concentration, but in high concentrations is nearly inversely proportional to it. Experiments 1 and 2 are not comparable as they were made with different enzyme concentrations. Each reaction by itself, however, was monomolecular. There is an

TABLE II.

Casein Concentration and Rate of Hydrolysis.

0°C., pH 8.2., M/3 borate buffer.

Experiment 1.				
Concentration of casein, *per cent*	3.5	1.75	0.87	
KE	0.071	0.13	0.29	
$KE \times$ casein concentration	0.25	0.23	0.21	
Experiment 2.				
Concentration of casein, *per cent*	1.36	0.68	0.34	0.17
KE	0.51	0.98	1.7	2.3
$KE \times$ casein concentration	0.70	0.66	0.58	0.39

indication that in very dilute casein solution the theoretical result would be obtained, but owing to experimental difficulties this cannot be shown conclusively. Similar results had been obtained by Taylor.[9]

The result is exactly analogous to the hydrolysis of cane-sugar by acid in that any one hydrolysis curve is monomolecular but the constants vary with the initial concentration of substrate. In the case of sugar hydrolysis the constants increase with increasing sugar concentration whereas here they decrease. This anomaly in the case of sugar hydrolysis, although the reaction is the classical example of a monomolecular reaction, has never been completely explained.

[9] Taylor, A. E., On fermentation, *Univ. Calif. Pub. Path.*, 1907, i, 239.

The opinion seems to be growing that it is due to an equilibrium between the water and sugar resulting in the formation of hydrated sugar molecules.[10]

The physical meaning of this result is that above about 1 per cent casein the actual amount hydrolyzed instead of the per cent hydrolyzed in a given time is independent of the casein concentration. This is a common result in enzyme reactions and has been usually ascribed to the formation of an intermediate compound. Experimental evidence has already been found, however,[11] which contradicts such an assumption and in any case such an assumption would predict rising velocity constants, during the course of the reaction. This is not the experimental result. We are forced to the conclusion, therefore, that the peculiarity is not due to any reaction between the enzyme and substrate, but between probably the substrate and the water, and also that the products of the reaction must be able to replace the casein in this equilibrium. This conclusion is further indicated by the fact that the effect on the constants depends solely on the relative casein-water concentration and not on the enzyme-casein concentration as might be expected if the equilibrium were between the enzyme and casein, although this evidence is not conclusive in itself. It may be pointed out that the term in the various equations which have been derived for enzyme hydrolysis and which has been supposed to represent an equilibrium between the substrate and enzyme could be interpreted just as well as representing an equilibrium between the substrate and water. The only difference would be as regards the physical significance of the constants. An equation of the same form, assuming a combination of the casein and water will accurately calculate the results shown in Table II, but such an equation contains two arbitrary constants and does not carry conviction in the absence of experimental evidence. It was stated by the writer in a preceding paper[11] that the discrepancy disappeared when the casein remaining in solution was used as the indicator. This result was obtained three times with a certain preparation of casein but has not been found again with any other casein preparations although the experiment

[10] Scatchard, G., *J. Am. Chem. Soc.*, 1921, xliii, 2387.

[11] Northrop, J. H., *J. Gen. Physiol.*, 1921–22, iv, 487; 1923–24, vi, 337.

has been repeated a number of times. The writer is undecided as to whether it was an experimental error or was due to some peculiarity of the casein in question. It is, perhaps, significant that, as will be shown later, the effect is not apparent with edestin, but is still more marked with gelatin.[11] Gelatin and casein have a relatively high viscosity whereas edestin has practically the viscosity of water. The viscosity itself, however, is not the cause as has already been shown. The effect is much too small, as may be seen from Table III, in which the rate of hydrolysis of two solutions is compared, to one of which sugar has been added. There is some effect, but not sufficient to account for the observed figures. Garrett and Lewis[12] have pointed out that if the reaction velocity is affected by the viscosity the reaction is probably between two molecules rather than the decomposition of one, since the latter could hardly be influenced by the viscosity.

TABLE III.

Effect of Viscosity.

	1 per cent casein solution.	1 per cent casein + 30 per cent sucrose.
Relative viscosity, (H_2O = 1.00)	1.2	3.5
K	0.24	0.13

It was pointed out above that the constancy of the velocity constants for any one reaction could only be accounted for supposing that the products of the reaction affected the velocity to the same extent as does the casein. An experiment was therefore carried out in which the casein solution was made up (*a*) with water, and (*b*) with a solution of digested casein. The result is shown in Table IV. It is evident that the digested casein has no effect. It must be remembered, however, that this solution of digested casein had been in contact with trypsin much longer than in the experiment itself, where the first products of the splitting of casein are present. It is quite possible, therefore, that the lack of effect is due to this difference. That this is actually the case may be seen from the following experiment.

[12] Garrett, W. H., and Lewis, W. C. M., *J. Am. Chem. Soc.*, 1923, xlv, 1091.

10 cc. of trypsin solution were added to 50 cc. of 1.2 per cent casein and 25 cc. of this solution added to 25 cc. of 1 per cent casein after varying lengths of time. The rate of hydrolysis of these solutions was then followed. All the solutions were at 0°C. and pH 8.4.

TABLE IV.

Effect of Digested Casein on Reaction Velocity.

	0.5 per cent casein.	0.5 per cent casein + 0.5 per cent digested casein.	1 per cent casein.
Velocity constant.....	0.98	1.02	0.54

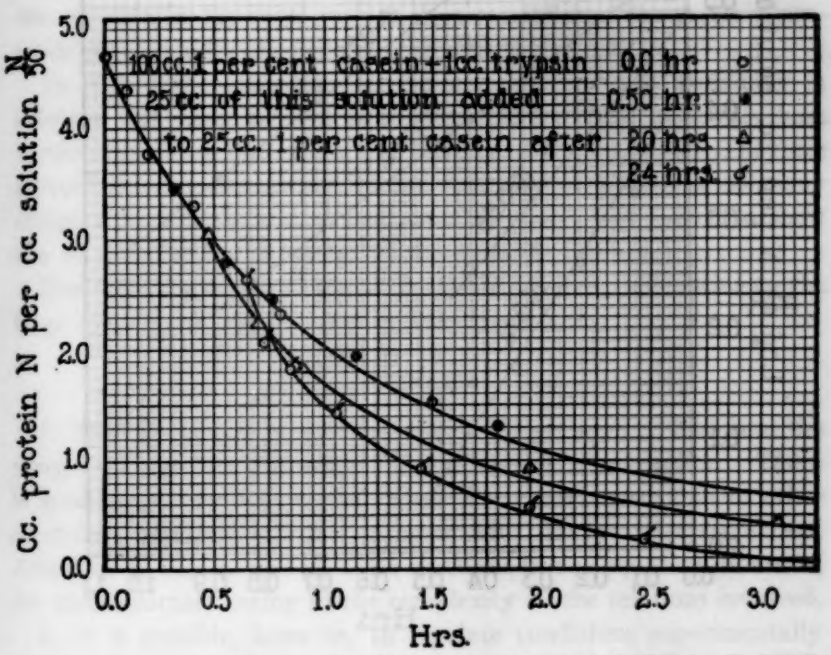

FIG. 2. Hydrolysis of 1 per cent casein solutions containing increasing proportions of digested casein.

Each solution, therefore, contained a total casein concentration of 1 per cent an increasing proportion of which, however, had been hydrolyzed before the experiment was begun. The result is shown in Fig. 2. In this figure the curves have been plotted so as to make

them coincide at the beginning. The second solution, for instance, at the beginning contained an equivalent of 3.45 cc. of casein. This point is therefore plotted on the first curve at 3.45 cc. and the time corresponding to this point considered as the starting time for the

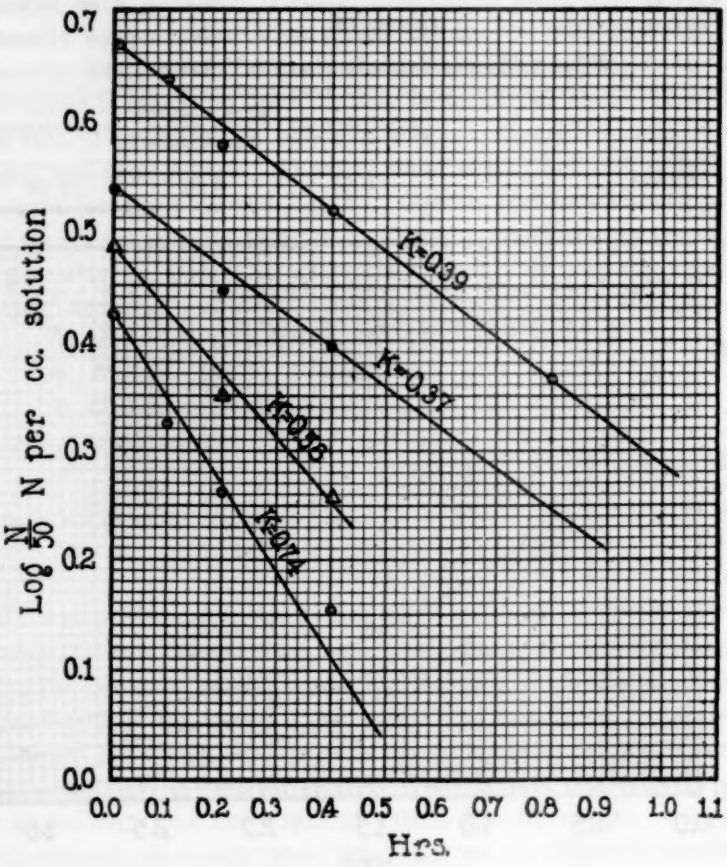

FIG. 3. Rate of hydrolysis of 1 per cent casein solution containing increasing proportions of digested casein.

second solution. It will be seen that all the solutions when plotted in this way are identical as regards casein and trypsin concentration at the first point. It might be expected, therefore, that the curves would all coincide and the figure shows that this is true of the first

two. The next two curves, however, go more rapidly and the last one (in which the casein had been acted on by the trypsin for 24 hours before adding to the fresh casein solution) is progressing at the same rate as though the trypsin had been added in water instead of in digested casein. The experiment shows that the first products formed have the same effect on the reaction as casein itself. That is, if the casein is diluted with a solution of digested casein which has just been formed, the anomalous effect of the substrate concentration disappears and the velocity constant is independent of the casein concentration as it should be. If, however, the digested casein solution has been in contact with trypsin for some time, it does not affect the reaction in the same way as does casein[13] and the reaction proceeds more rapidly the less concentrated the casein.

In Fig. 3 the logarithm of the amount of casein remaining in solution has been plotted against the time. The slope of these curves, therefore, represents the velocity of the reaction. They are somewhat irregular owing to the difficulty of obtaining so many samples accurately and almost simultaneously, but suffice to show the monomolecular character of the separate reactions.

The retarding effects of the products are not noticeable owing to the large amount of trypsin used in the experiment.

SUMMARY.

1. The kinetics of enzyme reactions diverge more or less from the simple mass action expression for a monomolecular reaction. There is good reason to believe that these discrepancies are due to other secondary reactions which also agree with the law of mass action. Attempts to incorporate all these reactions in one equation, however, are unsatisfactory owing to the complexity of the relations involved.

2. It is possible, however, to regulate conditions experimentally so that these secondary reactions are reduced to a minimum. This has been done in the case of trypsin digestion by working at a low temperature, which prevents inactivation of the trypsin, by using a

[13] A similar anomalous result, indicating the existence of consecutive reactions, has been observed by Simons in Nelson's laboratory (Simons, H. L., A study of the initial velocity in the hydrolysis of sucrose by invertase, Dissertation, Columbia University, New York, 1921).

large amount of trypsin, which prevents the inhibiting effect of the products from becoming noticeable, and by using the disappearance of the protein as the indicator which obviates the complicating effects of the consecutive reactions.

3. Under these conditions the hydrolysis, for any initial concentration of casein is accurately represented by the monomolecular formula, $\frac{dx}{dt} = KE\ (A-x)$. The effect of variations in the initial trypsin concentration are also correctly predicted.

4. If the initial casein concentration is varied, however, the value of the constant changes for each casein concentration, becoming less as the casein increases and eventually becoming nearly inversely proportional to the casein concentration. It is pointed out that this cannot be due to a compound between enzyme and casein, nor to the viscosity, but is probably owing to an equilibrium between the casein and water, in which the casein can be replaced by the first cleavage products. This is corroborated by the fact that if the casein is dissolved in a freshly prepared solution of digested casein, the anomalous effect of the substrate concentration disappears and the reaction is typically monomolecular in every respect. A solution of digested casein which has been in prolonged contact with trypsin does not have this effect.

5. It is pointed out that the various equations that have been proposed to account for the enzyme reactions on the basis of a compound between the enzyme and substrate could be applied equally well on the basis of a compound between water and the substrate which is attacked by the enzyme.

THE KINETICS OF TRYPSIN DIGESTION.

III. The Course of the Reaction with Constant Substrate under Conditions Causing Inactivation of the Enzyme.

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(Received for publication, December 18, 1923.)

It was shown in the preceding paper in this series that if experimental conditions are so chosen as to avoid a decrease in the amount of active trypsin either by spontaneous inactivation or inhibition by the products of digestion the reaction followed accurately the monomolecular formula. The present experiments were planned to show the course of the reaction when a single disturbing factor, the spontaneous inactivation of the enzyme, is at work. These conditions can be approximated by using a very concentrated solution of gelatin as substrate and carrying on the hydrolysis at 60°C., with a high concentration of trypsin. At this temperature, the enzyme is rapidly inactivated so that the reaction soon stops. The inhibiting effect of the products is reduced to a minimum since only a small amount of hydrolysis occurs and since a relatively large amount of trypsin is used. The percentage of the gelatin hydrolyzed is so small that the substrate concentration may be considered approximately constant.

The basic formula for the reaction is again

$$\frac{dx}{dT} = \text{K (substrate) (enzyme) (water)}$$

Since under these conditions the substrate does not change significantly the substrate concentration may be included with the water concentration in the constant so that the formula can be written

$$\frac{dx}{dT} = \text{K (enzyme)} \qquad (1)$$

Further, since the effect of the products on the enzyme is small, the enzyme concentration is decreasing as a function of time only. In order to integrate the formula it is necessary to know what this function is, but as a first approximation it is possible to compare the average reaction rate over a short time interval with the average amount of enzyme present during this interval. This procedure is, of course, not strictly correct, since it assumes that the reaction velocity is constant over the interval. The formula for this purpose is

$$\frac{\Delta x}{\Delta T} = \frac{x_2 - x_1}{T_2 - T_1} = \frac{C\,(E_{T_1} + E_{T_2})}{2}$$

in which x_1 is the amount of hydrolysis at time, T_1; x_2 the amount at time, T_2; E_{T1} the amount of the enzyme at time, T_1; and E_{T2} the amount of enzyme at time, T_2. The amount of enzyme must be determined by an independent experiment.

Experimental Procedure.

50 cc. of 11 per cent gelatin were titrated to pH 6.0 with NaOH (at this pH the action of trypsin causes no change in pH), placed in water bath at 60°C., 4 cc. of 10 per cent trypsin (Fairchild's) added, and the increase in the formol titration followed. 2 cc. samples were pipetted into 5 cc. of solution containing 3 cc. of 40 per cent formaldehyde and titrated to match a standard solution of about pH 8.5 N/50 alkali, using phenolphthalein as the indicator. The reaction is stopped by the formaldehyde. The standard used as the end-point was prepared by adding 1 drop of 0.1 per cent phenolphthalein to one of the samples and titrating to the maximum pink. 1 drop of 0.5 per cent phenolphthalein was added to the other samples and they were then titrated to match the standard. This procedure obviates all difficulty due to differences in color, etc., and allows a very accurate titration even with N/50 alkali. The total increase in formol titration under condition for the maximum action of trypsin has been found to be about 700 per cent.[1] Since in these experiments the original titration was about 5 cc., the reaction would have continued to at least 35 cc. before the substrate had been used up. The maximum obtained was 2 to 3 cc. increase so that as stated above the substrate concentration can be considered approximately constant.

Determination of the Relative Trypsin Concentration.

1 cc. samples were pipetted into 10 cc. of cold water and the amount of trypsin determined by noting the change of viscosity of a standard gelatin solution to

[1] Northrop, J. H., *J. Gen. Physiol.*, 1921–22, iv, 57.

which 0.2 cc. of this sample had been added as described by Hussey and the writer.[2] The figures given for the amount of trypsin are the reciprocal of the time in hours required to cause a 10 per cent change in the viscosity of the standard gelatin solution.

The result of an experiment performed in this way is shown in Fig. 1. It is evident that the trypsin is decreasing about as rapidly as the formol

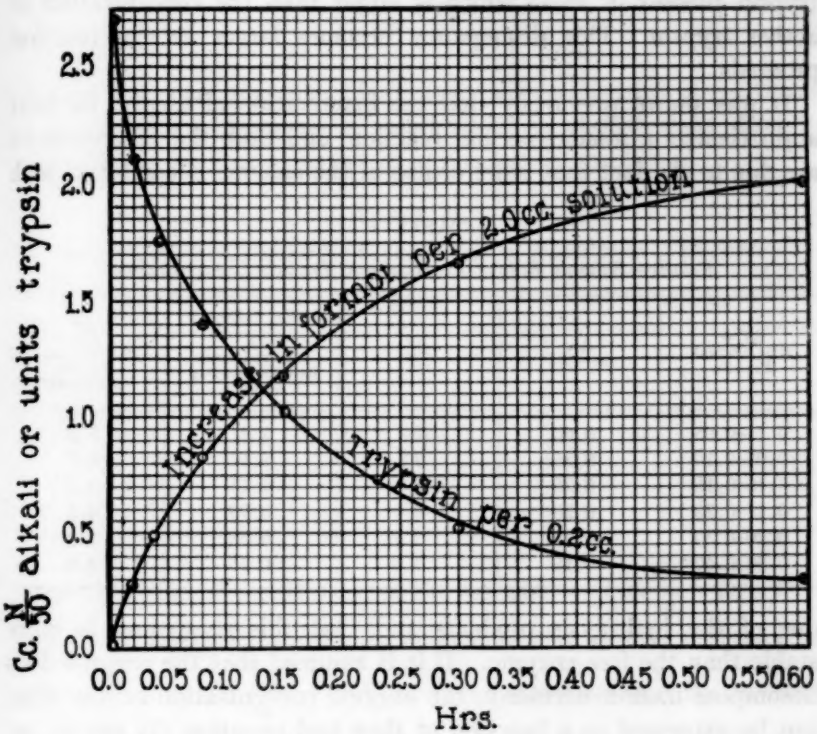

FIG. 1. Rate of digestion of gelatin and inactivation of the trypsin at 60°C.

titration figure is increasing. The relation between the rate of hydrolysis and the enzyme concentration interpolated from the curves is shown in Table I. The rate is closely proportional to the enzyme concentration as the theory demands. A drop in the constant is to be expected since two factors have been neglected, both of which affect the constant in the same way: first, the substrate concentration

[2] Northrop, J. H., and Hussey, R. G., *J. Gen. Physiol.*, 1922–23, v, 353.

is not constant, but is decreasing slightly; and second, the enzyme concentration as determined is larger than that actually present in the solution, owing to the fact that a portion of the enzyme is inactivated by the products of the reaction.[3] This inactivation is reversible and disappears when the solution is diluted as is necessarily done in the determination. The figure obtained is actually, therefore, the total trypsin present, a value which is larger than the concentration of active trypsin. This discrepancy becomes larger as the reaction proceeds.

It was shown previously that the inactivation of trypsin by heat approximates a monomolecular reaction[3] and that the discrepancies are due to the fact that more or less of the enzyme is combined with

TABLE I.

Relation Between Rate of Reaction and Trypsin Concentration.

ΔT = time interval, $T_2 - T_1$.	Δx, $x_2 - x_1$.	$\frac{\Delta x}{\Delta T}$	$\frac{E_{T_1} + E_{T_2}}{2}$, average trypsin for corresponding interval.	$\frac{\frac{\Delta x}{\Delta T}}{\text{trypsin concentration.}}$
0 –0.05	0.60	12	2.2	5.5
0.05–0.10	0.35	7	1.47	4.7
0.10–0.15	0.25	5	1.15	4.3
0.15–0.20	0.20	4	0.90	4.5
0.20–0.30	0.27	2.7	0.65	4.2
0.30–0.40	0.18	1.8	0.45	4.0

one of the hydrolysis products and that this compound is more stable than the free enzyme. If it is assumed that the enzyme does decompose monomolecularly, the enzyme concentration at any time can be expressed as a function of time and equation (1) can be integrated.[4] If E_o is the enzyme concentration at the beginning, the enzyme concentration at any time, T, is $E_o e^{-KT}$ in which e is the base of the natural logarithms. Equation (1) may therefore be written

$$\frac{dx}{dT} = C E_o e^{-KT}$$

[3] Northrop, J. H., *J. Gen. Physiol.*, 1921–22, iv, 261.

[4] *Cf.* Tammann, G., *Z. physiol. Chem.*, 1895, xviii, 426.

which on integration becomes

$$x = \frac{C E_o}{K}(1 - e^{-KT})$$

in which K is the constant for the decomposition of trypsin. Further,

when $T = \infty$

$$x = \frac{C E_o}{K} \tag{2}$$

That is, the total amount of hydrolysis will be directly proportional to the amount of enzyme—a result which is at first sight directly contradictory to that expected in any catalytic reaction.

TABLE II.

Monomolecular Constants for Digestion and for Inactivation of Enzyme.

Time.	K enzyme.	K digestion.
hrs.		
0.02	5.4	3.0
0.04	3.5	2.9
0.08	2.5	2.9
0.15	2.0	2.4
0.30	2.0	2.3
0.60	2.5	2.5

Let the value of x at the end of the reaction $= A = \frac{CE_o}{K}$, then $x = A - Ae^{-KT}$ which is the ordinary monomolecular formula and may be written $K = \frac{1}{T} \ln \frac{A}{A-x}$. K, however, is not the velocity constant for the hydrolysis of the gelatin but is the constant for the rate of inactivation of the enzyme, while A is not the total substrate present but the amount decomposed at the end of the reaction. The curves for the inactivation of the trypsin and for the hydrolysis of the gelatin shown in Fig. 1 should, therefore, both be approximately monomolecular and should both give the same value for the constant, K. That this is true is shown in Table II. The figures are as good as can be expected with the very short time intervals which must necessarily be employed. The gradual decrease is due to the pro-

tective action of the products of the reaction on the trypsin as explained above. The experiment shows that the decrease in rate of the reaction is simply due to the inactivation of the enzyme as was predicted from the theory and that if the enzyme could be kept constant also the time curve would be a straight line.

Effect of Varying the Initial Enzyme Concentration.

It was noted above that the velocity of the reaction depended solely on the amount of enzyme present at any time and that the constant for the reaction, therefore, represents the constant for the decomposition of the enzyme. If this is true the value of the constant should be independent of the concentration of enzyme, a result which is just the opposite of the usual catalytic reaction. In order to test this point the experiment was repeated with a series of different enzyme concentrations. The result is shown graphically in Figs. 2 and 3. In Fig. 3 the log of the amount of hydrolysis is plotted against the time so that if the reaction is monomolecular the resulting curves should be straight lines. If the velocity constants are the same, the lines should be parallel and the figure shows that this is approximately the case. In Fig. 2 the actual increases in the formol titration are plotted against the time. The experiment shows that at any time the amount of hydrolysis is proportional to the original enzyme concentration, while the time required to cause a constant amount of hydrolysis is not proportional to the enzyme concentration, but decreases more rapidly than the enzyme increases. This result has been obtained in other instances and has been used as evidence of the heterogeneous nature of the reaction. These experiments show, however, that it is simply due to the fact that the enzyme is decreasing as a function of time, instead of as a function of x. The final value reached is also proportional to the original enzyme concentration. This is the result predicted by equation (2) and is exactly the opposite of the result ordinarily obtained in enzyme or catalytic reactions; namely, that the velocity constant is proportional to the concentration of catalyst while the total amount of hydrolysis is independent of the concentration of catalyst. In these experiments the velocity constant is independent of the amount of trypsin and the total amount of hydrolysis directly proportional to it. If this experiment had been

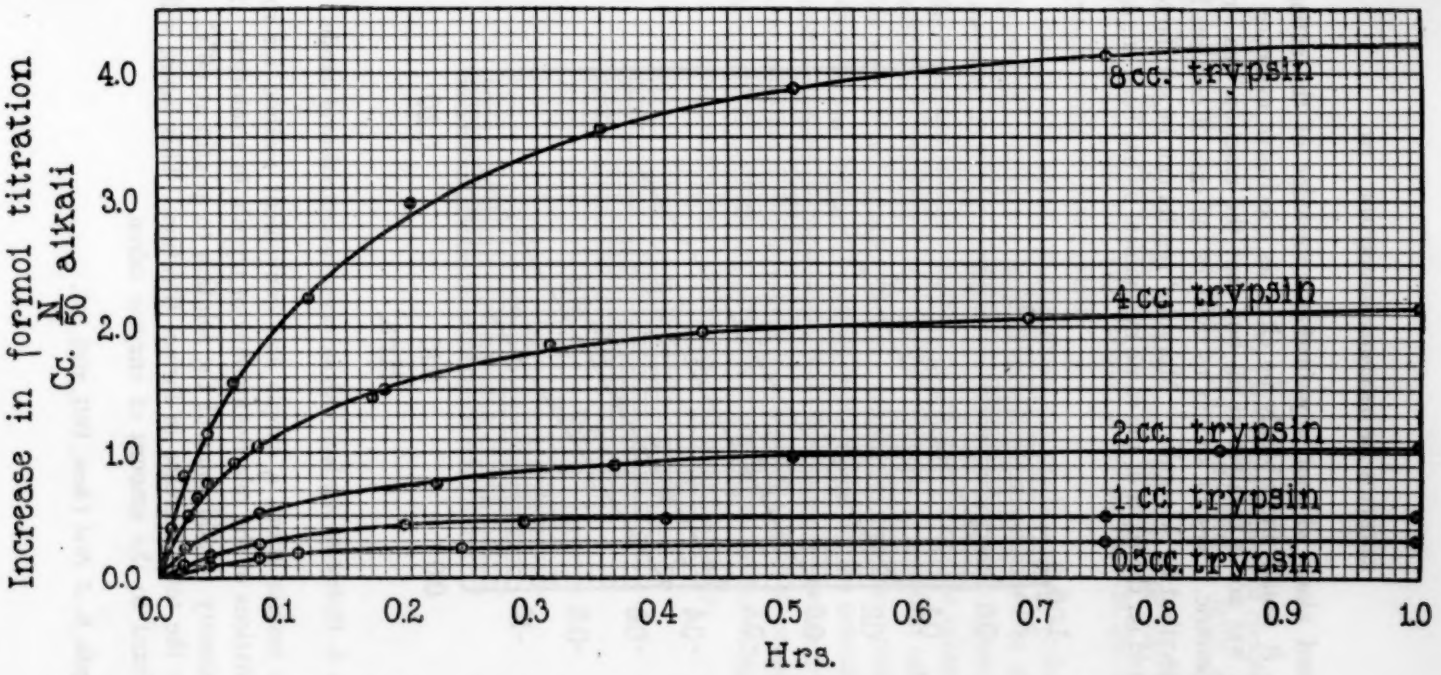

FIG. 2. Hydrolysis of gelatin with increasing concentrations of trypsin at 60°C.

performed alone without any further knowledge of the action of trypsin it would undoubtedly be cited as conclusive evidence that trypsin was not a catalyst at all but that the reaction was simply stoichiometric. Results similar to these in the case of catalase have been described by Morgulis.[5] Morgulis' results can be satisfactorily calculated on the basis of the same mechanism as has been employed here.

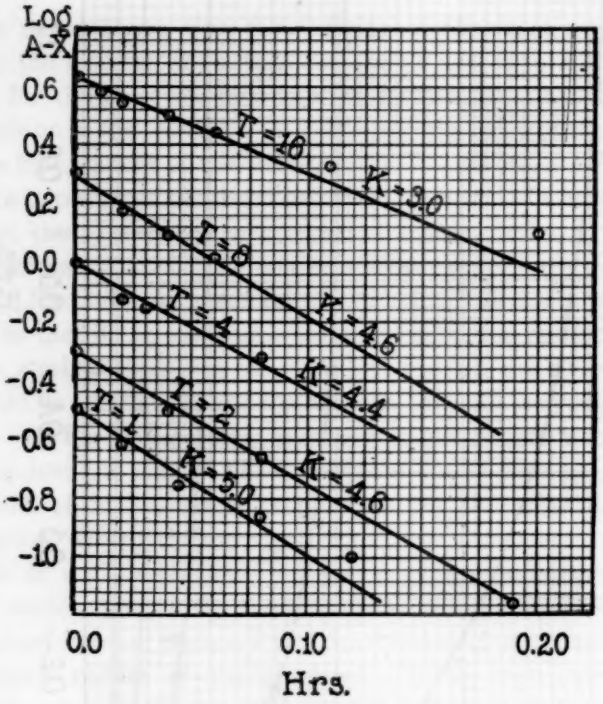

FIG. 3. Hydrolysis of gelatin with increasing concentrations of trypsin.

These results afford a simple and convenient method for rough determinations of the relative amount of trypsin in a solution. It is only necessary to add some of the solution to gelatin at 60°C., and measure the total increase in formol titration. This figure will be proportional to the amount of enzyme taken.

[5] Morgulis, S., *J. Biol. Chem.*, 1921, xlvii, 341.

SUMMARY.

1. A study has been made of the rate of hydrolysis of concentrated gelatin solutions at a high temperature and with a large amount of trypsin.

2. Under these conditions the substrate concentration may be considered constant and the only variable is the decrease in the amount of trypsin owing to inactivation.

3. The theory based on the mass law predicts that under these conditions (*a*) the rate at any time will be proportional to the concentration of trypsin at that time; (*b*) the reaction should approximate a monomolecular one if the total hydrolysis observed is taken as the amount of substrate available; (*c*) that the velocity constant calculated in this way should agree with the constant for the decomposition of the enzyme and that it should be independent of the concentration of enzyme instead of proportional to it as is usually the case; and (*d*) that the total amount of substrate decomposed should be proportional to the amount of trypsin added at the beginning instead of independent of it. These results have been obtained experimentally.

THE KINETICS OF TRYPSIN DIGESTION.

IV. The Course of the Reaction when Both Substrate and Enzyme Concentrations are Decreasing.

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(Received for publication, December 18, 1923.)

Under the conditions generally employed for tryptic digestion, the enzyme is more or less rapidly inactivated. It was shown in the preceding paper of this series that if the substrate concentration were made so large that it could be considered constant, the rate of reaction was controlled solely by the rate of inactivation of the enzyme. The present paper is the result of experiments in which the substrate concentration is also changing. In addition to the heat inactivation of the enzyme mentioned above there is an inhibition of its action due to the products of the reaction. In order to reduce this to a minimum, it is necessary to work with a large amount of trypsin so that the per cent inactivated by the products will be small. A low concentration of substrate also minimizes this effect. These conditions can be approximated by using edestin as substrate at 40°C. and in the presence of a high concentration of salt. The salt increases the solubility of the protein and at the same time decreases the activity of the trypsin and so allows the use of high enzyme concentrations without at the same time causing the reaction to proceed too rapidly for measurement.

The Time Course of the Reaction.

Experimental procedure.—5.0 gm. of edestin were dissolved in 50 cc. of M/10 phosphate buffer, pH 7.0, made up in 1 M NaCl. The solution was filtered at 70°C., cooled to 40°, and 10 cc. of 10 per cent Fairchild's trypsin added. 5 cc. samples were pipetted into 15 cc. of 7.5 per cent

trichloroacetic acid. The precipitate was removed by centrifuging, washed in trichloroacetic and dissolved in alkali and made up to 20 cc. Kjeldahl determinations were made on 5 cc. of this solution. 1 cc. samples were also removed into 10 cc. of cold water and the trypsin determination[1] made on 0.2 cc. of this solution.

The result of such an experiment is shown in Fig. 1. The initial trypsin concentration (for convenience in plotting) has been made to coincide with the initial N value by calculating the trypsin to 0.01 cc.

The experiment shows that the rate of hydrolysis of the edestin decreases very rapidly, much more so than would be expected from the simple monomolecular formula. The trypsin is also being rapidly inactivated and it is evident that no formula would hold even approximately which assumed the enzyme to be constant throughout the course of the experiment. It would be necessary to insert into the equation the amount of enzyme present at the time interval under consideration. This can be done by calculating the monomolecular constant for short time intervals and inserting the value of E found experimentally for the same time interval. This method is an approximation only since the value of E is not really constant even over short time intervals, and also because the amount of trypsin found is larger than the active trypsin present in the solution and combined with the products of the reaction. This trypsin is inactive in the original solution but active in the determination owing to the dissociation of the compound by dilution.[2]

Table I contains a summary of the results obtained in this way. Column 2 is the monomolecular constant for the reaction calculated from "point to point." This calculation corrects for the change in the substrate concentration and if there were no other variable, the resulting values should be constant. The table shows, however, that the values are decreasing very rapidly. If the theory is correct, this decrease must be due to the decrease in the amount of enzyme present so that if the value of the "constant" is divided by the concentration of trypsin present at the corresponding time the resulting figures should be constant. This is the case as is shown in the last column.

[1] Northrop, J. H., and Hussey, R. G., *J. Gen. Physiol.*, 1922–23, v, 353.
[2] Northrop, J. H., *J. Gen. Physiol.*, 1921–22, iv, 245.

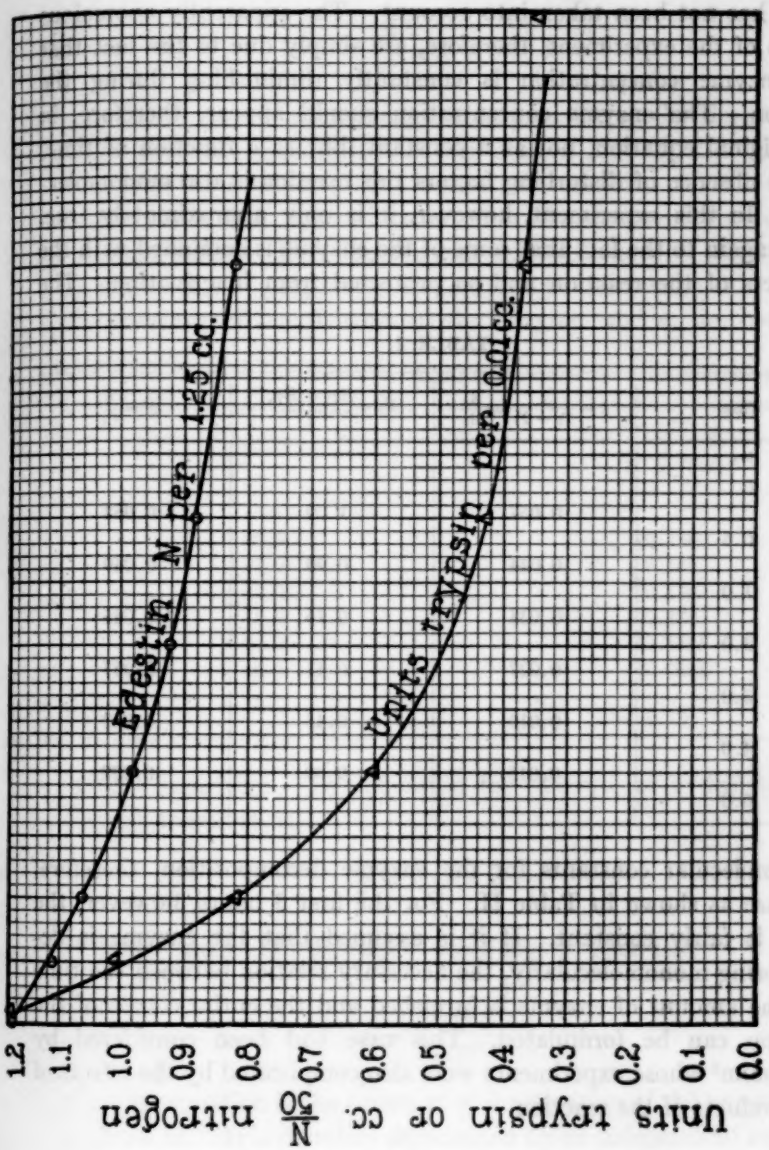

FIG. 1. Digestion of edestin and inactivation of trypsin at 40°C. pH 7.0.

There is still a slight drop due to the inhibiting effect of the products which has not been taken into account. The apparently anomalous results of the experiment, therefore, are simply due to the fact that the enzyme concentration is constantly diminishing during the reaction. The enzyme concentration should appear, therefore, in the original equation, not as a constant, but as a function of time. In the absence of disturbing factors this reaction is also monomolecular.[3] In this experiment, however, it is only approximately true, owing again to the fact that some of the enzyme is combined with the products of the reaction and so protected from inactivation. The

TABLE I.

Time.	$k = \frac{1}{T_2 - T_1} \log \frac{A_1}{A_2}$	$E_f = \frac{E_{T_1} + E_{T_2}}{2}$	$k' = \frac{k}{E_f}$
hrs.			
0			
	0.057	1.09	0.052
0.5			
	0.044	0.90	0.049
1.0			
	0.034	0.76	0.045
2.0			
	0.027	0.57	0.047
3.0			
	0.019	0.45	0.042
4.0			
	0.015	0.39	0.039
6.0			

monomolecular constants for the enzyme decomposition, therefore, decrease as shown in Table II. For the first 3 hours, however, the value is fairly constant. If it is assumed that the enzyme is decomposing monomolecularly, the necessary relation between the time and the amount of enzyme is furnished and the entire course of the reaction can be formulated. This case had been considered by Tammann[4] whose experiments were also complicated by the effects of the products of the reaction.

[3] Northrop, J. H., *J. Gen. Physiol.*, 1921–22, iv, 261.

[4] Tammann, G., *Z. physiol. Chem.*, 1895, xviii, 426.

Under these conditions the equation becomes

$$\frac{dx}{dt} = K(A - x)E_t$$

in which $A - x$ is the substrate concentration at the time, t, and E_t is the enzyme concentration at the time, t, instead of a constant as would be the case for a simple catalytic reaction. If it is assumed further as a first approximation that the enzyme is decomposing monomolecularly, then the enzyme concentration at any time will be $E_t = E_o e^{-Kt}$, E_o being the enzyme concentration at 0 time, K, the constant of inactivation of the enzyme, and e, the base of the

TABLE II.

Time.	$E = 1.2$ $K = \frac{1}{T} \log \frac{E_o}{E_t}$
hrs.	
0.5	0.158
1.0	0.165
2.0	0.150
3.0	0.130
4.0	[0.114]
6.0	[0.085]
Average K	= 0.15

natural logarithm. The equation for the rate of hydrolysis of the edestin, therefore, is

$$\frac{dx}{dt} = k_r(A - x)E_o e^{-Kt}$$

which on integration becomes (using logs to base 10)

$$k_r = \frac{K \log \left(\frac{A - x}{A}\right)}{E_o(10^{-Kt} - 1)} \quad (1)$$

K in the equation is the constant of inactivation of the enzyme shown in Table II. It is therefore determined by an independent experiment and is not simply an arbitrary constant. The values for the reaction constant k_r are given in Table III. They are as satisfactory as could

be expected when the approximate nature of the assumptions on which the equation is based are taken into account. The disturbing factor is the decomposition of the enzyme which does not follow the simple monomolecular reaction rate as assumed in deriving this equation. These irregularities can be accounted for as has been shown but cannot be taken into account mathematically in any simple way.

Fig. 2 and Table IV show the results of a similar experiment in which, however, the pH was 9.5. Trypsin is much more rapidly inactivated at this pH as is shown by the fact that the value of K_t is now 1.4 instead of 0.15. The reaction therefore comes to a standstill very much more quickly than in the first experiment.

There are several conclusions to be derived from this equation which at first sight are surprising. For instance if t is taken as infinity, equation (1) becomes

$$\frac{x}{A} = 1 - 10^{-\frac{k}{K}E}$$

In order to make the reaction complete, therefore, it would be necessary to have an infinite enzyme concentration for an infinite time, even neglecting any reverse reaction. It would seem that this conclusion would hold even though the relation of the enzyme to the time is not accurately expressed by the monomolecular formula. This was pointed out by Tammann.

If x is considered negligible to A so that the term $A - x$ can be considered a constant over the period of the reaction, the equation can be reduced to the form

$$C = \frac{1}{t} \log \frac{B}{(B - y)}$$

which is again monomolecular but with respect to the enzyme and not to the substrate since C is the inactivation constant of the enzyme and B the total amount of substrate decomposed and not the total amount present. This equation can be experimentally verified as was shown in the preceding paper.[5] If the substrate concentration is kept constant but the initial enzyme varied, there is no simple relation between either the amount of hydrolysis at a given time or the time

[5] Northrop, J. H., *J. Gen. Physiol.*, 1923–24, vi, 429.

TABLE III.

Value of $k_r = \frac{K}{E_o} \log \frac{\frac{A-x}{A}}{(10^{-Kt}-1)}$

$K = 0.15$ $\qquad \frac{K}{E_o} = 0.125$

$E = 1.2$

$A = 1.19$

Time	k_r
hrs.	
0.5	0.187
1.0	0.172
2.0	0.17
3.0	0.175
4.0	0.173
6.0	0.182

TABLE IV.

Hydrolysis of Edestin at 40°C. pH 9.5. M/10 Na_2CO_3, 1 M *NaCl.*

Time.	$A-x$	$k = \frac{1}{T_1 - T_2} \log \frac{A_1}{A_2}$	$E_t = \frac{E_{T_1}+E_{T_2}}{2}$	$k' = \frac{k}{E}$	$K_d = \frac{1}{t} \log \frac{E_o}{E}$	$k_r = \frac{Kd}{E_o} \log \frac{\frac{A-x}{A}}{(10^{-Kt}-1)}$
hrs.						
0	4.60					
		0.70	3.6	0.19		
0.10	3.92				1.46	0.15
		0.37	2.55	0.145		
0.20	3.6				1.40	0.13
		0.34	1.9	0.18		
0.30	3.33				1.39	0.12
		0.24	1.4	0.17		
0.40	3.15				1.38	0.12
		0.20	1.05	0.19		
0.50	3.02				1.34	0.12
		0.15	0.82	0.18		
0.60	2.92				1.30	0.12
		0.13	0.65	0.20		
0.80	2.75				1.05	0.13
		0.08	0.50	0.16		
1.60	2.40				0.80	0.16
Average					1.4	

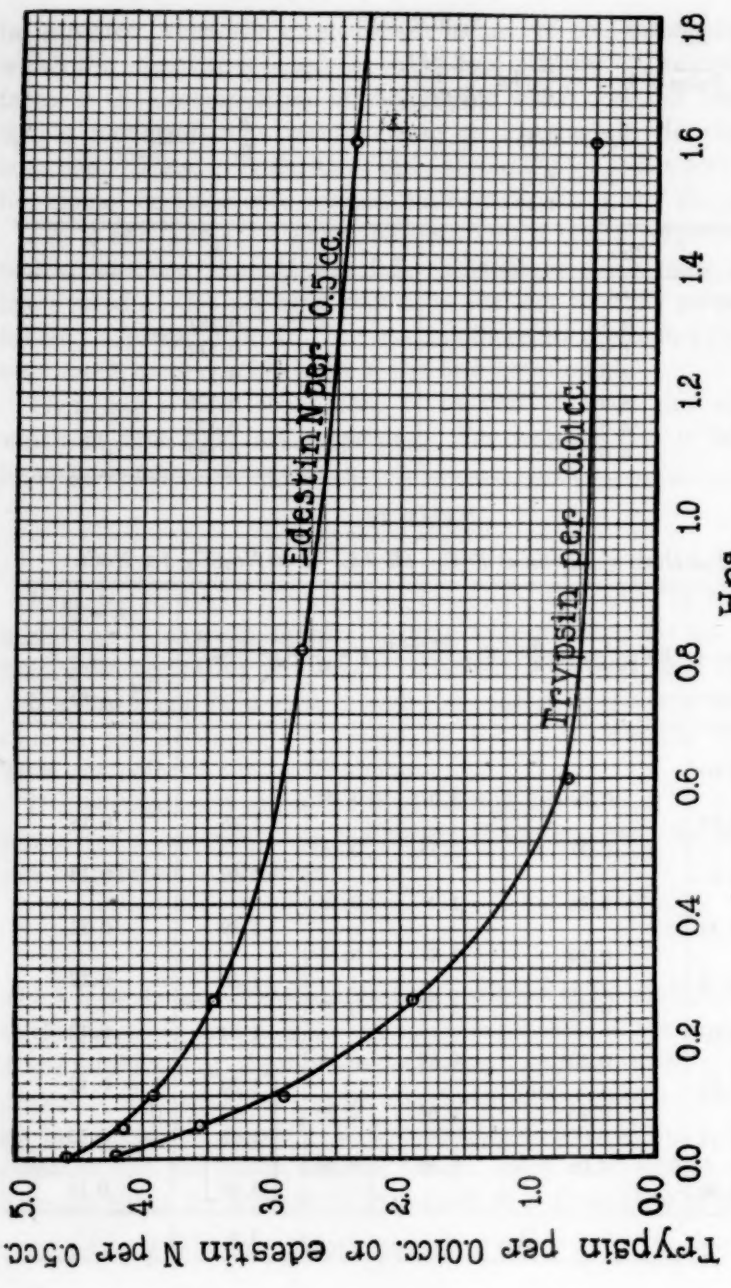

FIG. 2. Digestion of edestin and inactivation of trypsin at 40°C. pH 9.5.

required to form a given amount of hydrolysis. This is due to the fact that the rate of the reaction is a function of both time and of x. It has been frequently pointed out that in comparing the two reactions it is necessary to make the comparison over corresponding parts of the curve. Osterhout[6] and others recommended that the time required to cause a certain effect is a better measure than the effect produced after a certain time. This is probably usually true but there is no reason to predict *a priori* that one method will be better than the other. If the rate of the reaction is decreasing as a function of time then the comparison should be made at equal times. If it is decreasing as a function of the extent of the reaction then the comparison should be made at corresponding stages of the reaction. Failure to give a simple ratio by either method, however, does not prove that the reaction is contrary to any simple mass action formula, as is shown above.

Effect of Varying the Initial Substrate Concentration.

Equation (1) predicts that if the substrate concentration is varied the amount decomposed at any time will be in proportion to the original concentration. There will be no simple relation between the times required to cause any given amount of hydrolysis and the initial concentration.

The result of an experiment with different edestin concentrations is shown in Fig. 3 and Table V. The amounts hydrolyzed are in proportion to the original concentrations as predicted above. The reaction is therefore perfectly normal as far as the concentration of edestin is concerned. The time course of this reaction is fairly well represented by equation (1) also.

The Effect of Varying the Enzyme Concentration.

The digestion of an edestin solution with varying amounts of trypsin is shown in Fig. 4 and the corresponding enzyme concentration in Fig. 5. It is noticeable at once that the concentrated enzyme solutions are being much more rapidly inactivated than the dilute. This is the result of the protective action of the products of hydrolysis which would be more marked with a small amount of enzyme than

[6] Osterhout, W. J. V., *Science*, 1918, xlviii, 172.

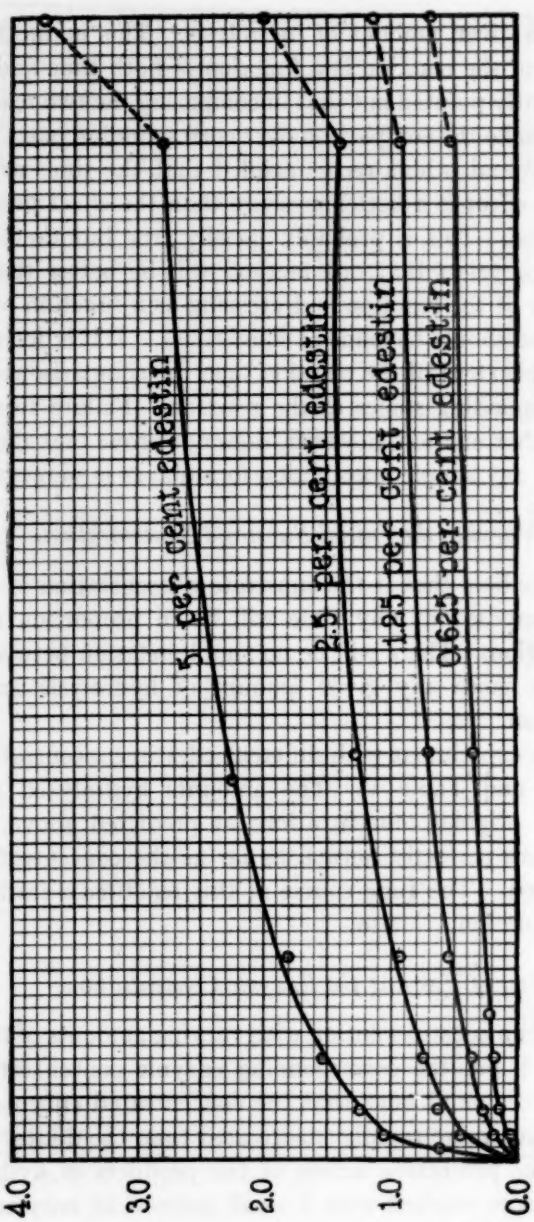

FIG. 3. Rate of digestion of different concentrations of edestin with constant trypsin concentration.

with a large amount as is seen to be the case. That is, with the smaller amounts of enzyme the effect of the products cannot be neglected as was done in the other experiments. The approximations used before, therefore, will not hold and equation (1) cannot be used.

TABLE V.

Amount of Edestin Hydrolyzed at Different Times with Varying Initial Concentrations of Edestin and Constant Trypsin. Edestin Hydrolyzed as Cc. N/50 N.

Time.	Initial edestin concentration in per cent.			
	0.62	1.25	2.5	5.0
hrs.				
0.5	0.15	0.30	0.60	1.30
1.0	0.22	0.43	0.80	1.60
2.0	0.28	0.59	1.05	2.00
4.0	0.37	0.75	1.35	2.40
8.0	0.50	0.95	1.45	2.80
20.0	0.70	1.15	2.00	3.75

TABLE VI.

Value of $k = \frac{1}{(T_1 - T_2)(E_1 + E_2)} \log \frac{A_1}{A_2}$ with varying values of E.

Time.	Relative amount of E at beginning.			
	1	2	4	8
hrs.				
0 –0.1	0.00125	0.0013	0.0013	0.0013
0.1–0.2	0.0013	0.0013	0.0008	0.0007
0.2–0.3	0.0013	0.0011	0.0011	0.0007
0.3–0.4	0.00125	0.0016	0.0011	0.0008
0.4–0.6	0.00138	0.00126	0.0008	0.00065
0.6–0.8	0.0014	0.00123	0.00108	0.0007
0.8–1.0	0.00145	0.00137	0.0007	0.0006
1.0–1.2	0.0015	0.00127	0.0010	0.00065
1.2–1.5	0.0015	0.00138	0.0011	0.00057
1.5–2.0	0.0014	0.00104	0.0010	0.00074

It is only possible to correct the observed rate by the amount of enzyme found experimentally at the corresponding times. This has been done in Table VI. The table shows that the anomalous results are due to this destruction of the enzyme and not to any peculiarity

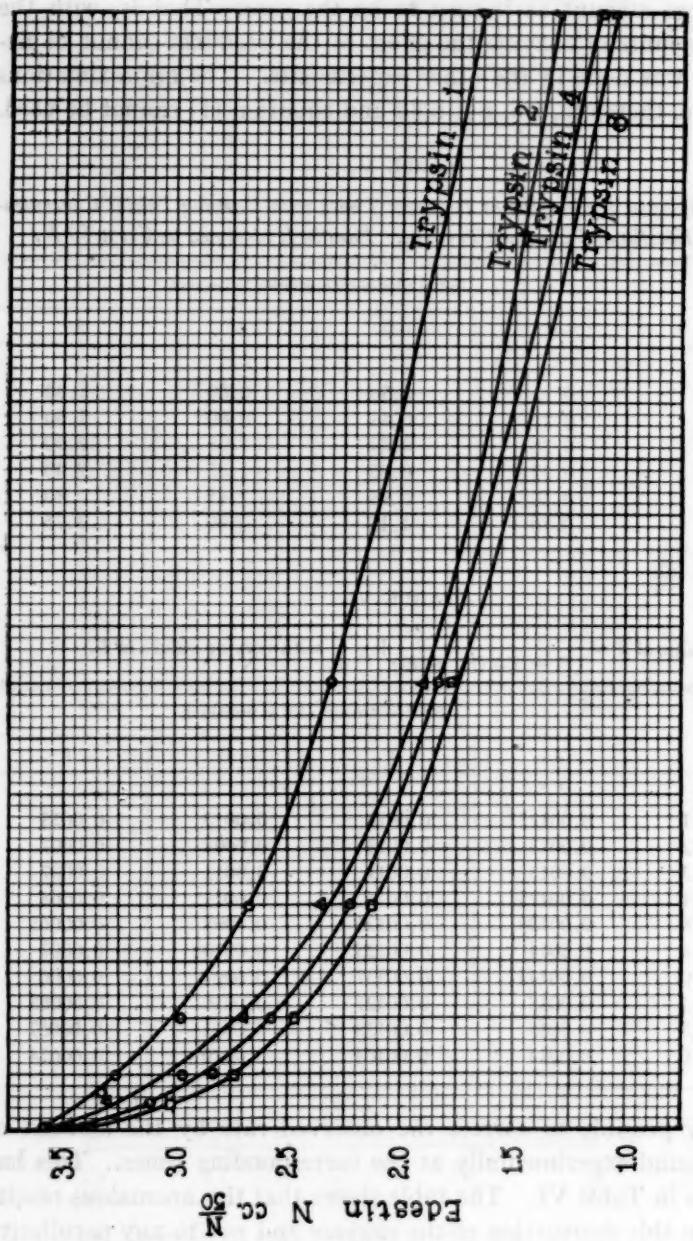

FIG 4. Rate of digestion of edestin with increasing concentration of trypsin.

of the reaction itself as the result of the edestin analyses alone would indicate. The figures for the trypsin concentration again contain the amount combined with the products of hydrolysis and this effect causes the drop in the constant as the trypsin concentration increases.

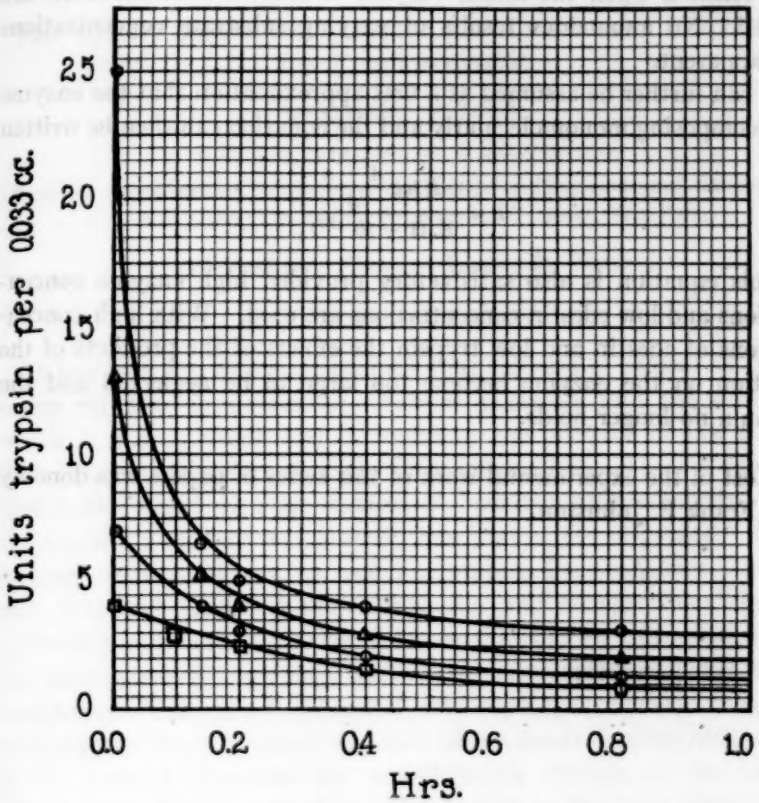

FIG. 5. Inactivation of trypsin during reaction plotted in Fig. 4.

SUMMARY.

The rate of hydrolysis of edestin by trypsin at 40° and in the presence of 1 M NaCl has been studied. Under these conditions the enzyme is rapidly inactivated and the equation for the reaction may be written

$$k = \frac{1}{(T_1 - T_2)\,E_1} \log \frac{A_1}{A_2}$$

in which E_t is the concentration of enzyme during the interval (T_1-T_2). This equation has been tested by determining the enzyme concentration at various times during the reaction and substituting these values in the above equation. The experimental results agree with this formula when the initial enzyme or edestin concentrations are varied. No anomalous results of varying substrate concentrations are apparent.

It can further be assumed as a first approximation that the enzyme is decomposing monomolecularly and the equation can then be written

$$k = \frac{K \log \frac{A - x}{A}}{E_o (1 - 10^{-Kt})}$$

This equation is also satisfactory provided high enzyme concentrations and low edestin concentrations are used. With high concentrations of edestin and low trypsin the effects of the products of the reaction on the enzyme become too large to be neglected and the formula no longer holds.

Most of the experimental work of this series of papers was done by Mr. Frank F. Johnston.

ON THE DIFFUSIBILITY OF THE CALCIUM OF BLOOD SERUM THROUGH COLLODION MEMBRANES.

The Effect of Sodium Chloride and Changes in Hydrogen Ion Concentration.

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(Received for publication, January 20, 1924.)

INTRODUCTION.

It has been generally maintained that calcium is present in blood serum in two forms one of which is diffusible and the other of which is non-diffusible and is bound to the proteins of the serum. This idea was first expressed by Pribram[1] in 1871. Later Rona and Takahashi[2] found with the method of "compensatory dialysis" that the serum Ca content was greater than that of the dialysate. Their findings were confirmed by von Meysenbug, Pappenheimer, Zucker, and Murray,[3] Cushny,[4] and Neuhausen and Pincus[5] found in experiments on pressure dialysis of serum that the Ca concentration of the dialysate was lower than that of the serum remaining within the membrane. Salvesen and Linder[6] have recently concluded from determinations made on blood sera and edema fluids that the differences in Ca content represent the non-diffusible calcium of the blood. About 25 to 50 per cent of the blood calcium has been thought by various investigators to be non-diffusible.

[1] Pribram, R., *Ber. köngl. Sächs. Ges. Wissensch.*, 1871, xxiii, 279.

[2] Rona, P., and Takahashi, D., *Biochem. Z.*, 1911, xxxi, 336.

[3] von Meysenbug, L., Pappenheimer, A. M., Zucker, T. F., and Murray, M. F., *J. Biol. Chem.*, xlvii, 1921, 529.

[4] Cushny, A. R., *J. Physiol.*, 1919–20, liii, 391.

[5] Neuhausen, B. S., and Pincus, J. B., *J. Biol. Chem.*, 1923, lvii, 99.

[6] Salvesen, H. A., and Linder, G. C., *J. Biol. Chem.*, 1923–24, lviii, 617.

It is the purpose of this paper to describe the effect of varying NaCl concentrations and pH on the diffusibility of serum calcium. In our experiments the amount of fluid used in dialysis was large enough to obviate any possible Ca membrane equilibrium condition between serum and fluid whereas in previous experiments, free diffusion has been impossible as a result of the methods used.

EXPERIMENTAL.

Blood was obtained from patients suffering from cardiac insufficiency, emphysema, or acute nephritis. The blood was permitted to clot and the serum was later separated by centrifugalization. In each series of experiments the same lot of serum was used through-

TABLE I.

Influence of NaCl on the Diffusibility of the Ca in Blood Serum through Collodion Membranes at pH 7.4. (Ca Calculated in Mg. Per 100 Cc. of Serum.)

No.	Dialysis time.	Ca of serum before dialysis.	Ca of serum after dialysis against varying concentration of NaCl.			
			0.0 per cent NaCl.	0.2 per cent NaCl.	0.4 per cent NaCl.	0.8 per cent NaCl.
	hrs.					
1	48	9.4	3.8			0.1
2	60	7.9	2.2	1.1	0.6	0.0
3	44	9.9	2.2	0.9	0.2	0.0
4	44	10.1	4.7			0.2

out. For dialysis, serum was placed in stoppered collodion sacs (made with Merck's U.S.P. non-flexible collodion, concentrated about 30 per cent) molded in test-tubes of about 20 cc. capacity. The sacs containing serum were floated in 2 liters of the fluid to be used for dialysis and when the experiment continued for more than a day, the dialysate was renewed at the end of 24 hours. Dialysis was carried on at about 10°C. to inhibit bacterial growth. The pH measurements were made by means of the colorimetric method and were only made on the dialysates. Sodium bicarbonate and HCl were used to control the hydrogen ion concentration. Calcium determinations were made by the method of Kramer and Tisdall.[7] Protein concentration was controlled

[7] Kramer, B., and Tisdall, F. F., *J. Biol. Chem.*, 1921, xlvii, 475.

in all experiments but the results are not presented as the slight changes observed were not significant. The results of dialysis of serum against varying concentrations of NaCl at pH 7.4 are presented in Table I. Table II shows the influence of pH variation on the diffusibility of Ca when serum is dialyzed against distilled water.

TABLE II.

Influence of pH on the Diffusibility of Blood Serum Calcium. (Ca Calculated in Mg. Per 100 Cc. of Serum.)

No.	Dialysis time.	Ca of serum after dialysis against distilled water.		Ca of serum before dialysis.
		pH 7.4	pH 2.5	
	hrs.			
1	16	2.2	0.0	7.9
2	16	2.2	0.1	9.9
3	44	4.7	0.0	10.1

DISCUSSION.

It may be seen from Table I that at the pH of the blood *all* of the serum Ca is diffusible through a collodion membrane when the dialysis takes place in the presence of a large volume of 0.8 per cent NaCl. It may furthermore be noted that in the absence of NaCl, about 25 to 45 per cent of the Ca is non-diffusible. In other words, NaCl seems essential for the complete diffusibility of serum Ca at a pH of 7.4. Associated with the decreasing concentrations of NaCl and increasing retention of Ca within the collodion membranes, increasing precipitation of globulin was quite naturally observed. An analogous observation was made by J. Loeb[8] on the diffusion of potassium through the membrane of the egg of *Fundulus*. J. Loeb suggested that the diffusibility of potassium might in some way be related to the solubility of globulin in NaCl solutions. This suggested the possibility that on the acid side of the isoelectric point of the serum proteins, the Ca of serum should be completely diffusible even in the absence of NaCl. Table II shows this to be the case at a pH of 2.5.

[8] Loeb, J., *J. Biol. Chem.*, 1917, xxxii, 147.

The diffusibility of part of the Ca against distilled water at pH 7.4 may only be apparent and may result from the fact that the initial NaCl concentration of the serum is great enough to allow some calcium diffusion. This can only be definitely proven by using pure protein solutions.

CONCLUSIONS.

1. Serum calcium is *completely* diffusible through collodion membranes in 0.8 per cent NaCl at a pH of 7.4.

2. About 55 to 75 per cent of serum Ca is diffusible through collodion membranes in distilled water at a pH of 7.4.

3. Serum Ca is completely diffusible when dialyzed against HCl at a pH of 2.5.

THE ISOELECTRIC POINT OF GELATIN AT 40°C.

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(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, January 18, 1924.)

In order to explain certain experiments on the swelling of gelatin, together with other data collected from the literature, Wilson and Kern[1] suggested that gelatin exists in two forms having different isoelectric points. At temperatures above 35°C. they assumed that gelatin exists only in a "sol form" having its isoelectric point at pH 7.7, while at lower temperatures it exists as mixtures of this with the "gel form" having its isoelectric point at pH 4.7. The change to the "sol form" was supposed to be favored by increase in alkalinity or in temperature. In order to test the validity of their idea, the following experiments have been carried out.

Measurements were made of the osmotic pressure of 1 per cent gelatin solutions at 40°C., by a method similar to that used by Loeb.[2] The gelatin was purified by washing at the isoelectric point as described by Loeb.[3] Solutions containing 1 per cent of gelatin and varying amounts of sodium hydroxide or hydrochloric acid were placed in collodion test-tubes holding about 15 cc. Each tube was fitted with a rubber stopper and a glass manometer tube, and was submerged in 250 cc. of distilled water in a wide-mouthed Erlenmeyer flask closed by a two-hole rubber stopper. The outside air was excluded by connecting the top of the manometer tube with the air space in the flask by means of rubber and glass tubing. The flasks were put in a water bath at 40° ± 0.05°, and left about 17 hours for the attainment of equilibrium. The osmotic pressure was measured

[1] Wilson, J. A., and Kern, E. J., *J. Am. Chem. Soc.*, 1922, xliv, 2633; 1923, xlv, 3139.

[2] Loeb, J., Proteins and the theory of colloidal behavior, New York and London, 1922, 66.

[3] Loeb,[2] p. 35.

in terms of millimeters of the solution in the tubes, and the pH of the inside and outside solutions was measured with hydrogen electrodes at 25°C., or, in some cases, colorimetrically. The results are given in Table I and Fig. 1.

Fig. 1 shows that near pH 4.7, the accepted isoelectric point of gelatin, the osmotic pressure is at a minimum, while near pH 7.7 it has high values. Moreover, in Table I the pH values of the inside solutions are higher than those of the corresponding outside solutions only when the inside solutions are more acid than pH 4.7, the reverse being true on the alkaline side of pH 4.7. According to the theory

TABLE I.

Osmotic Pressure of 1 Per Cent Gelatin Solutions at 40°C.

Osmotic pressure.	pH inside.	pH outside.
mm.		
164	4.4	4.1*
72	4.6	4.2*
29	5.0	5.3*
147	5.5	6.6*
283	6.2	6.5*
324	6.4	7.2*
376	7.6	8.7
379	8.3	9.0
410	9.0	9.4
403	9.0	9.8
402	9.2	10.0

* Colorimetric measurement.

of membrane equilibria[4] this change in sign of the difference in pH between the inside and outside solutions must occur at the isoelectric point of the non-diffusible ampholyte. Hence these results are incompatible with the idea that gelatin at 40° exists in a form isoelectric at pH 7.7.

Since the pH measurements had been made at 25°, while the osmotic pressure had been measured at 40°, a comparison was made of the titration curves of gelatin with sodium hydroxide at these two temperatures. Hydrogen electrode measurements were made in

[4] Loeb,[3] p. 137.

the titrating vessel described by Bovie,[5] with the results shown in Fig. 2. The points fall practically on one curve below pH 8.

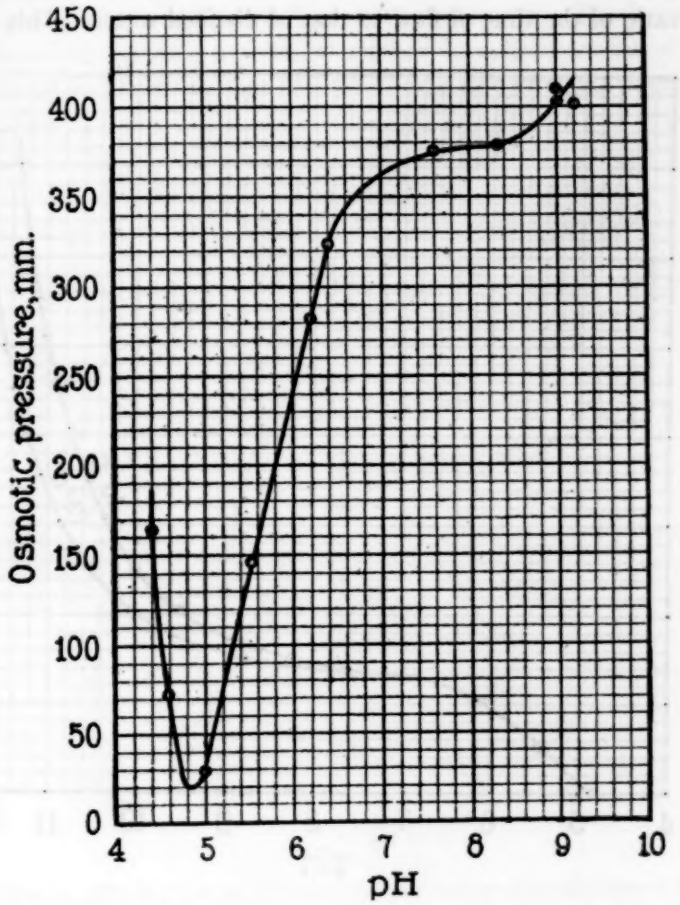

FIG. 1. Effect of pH on osmotic pressure of 1 per cent gelatin solution at 40°C.

Measurements were also made at 40° of the viscosity of 1 per cent solutions of gelatin containing varying amounts of hydrochloric acid or sodium hydroxide. The solutions were prepared by diluting a stock solution of isoelectric gelatin, melted by heating with warm

[5] Bovie, W. T., *J. Am. Chem. Soc.*, 1922, xliv, 2892.

water not above 45°C., and after mixing were kept about 2 hours in the thermostat at 40°C. before measurements were made. The viscosity was determined with Ostwald viscometers, and is expressed as the ratio of the time of flow to that of distilled water. This neg-

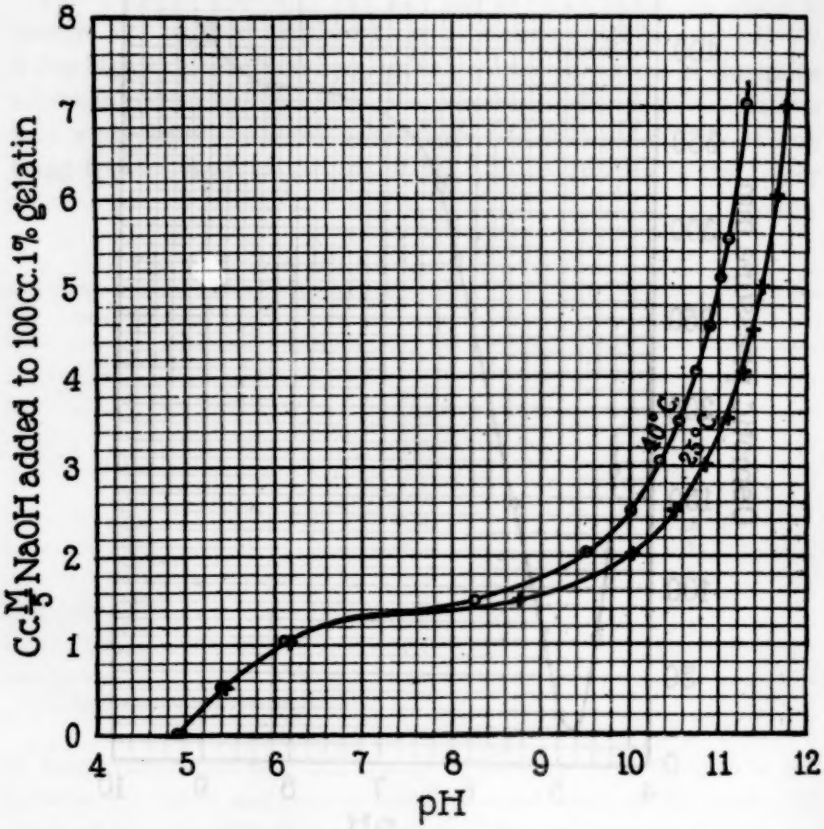

FIG. 2. Titration of 1 per cent gelatin solutions with NaOH at 25° and at 40°C.

lects the difference in density between water and a 1 per cent gelatin solution, which, according to Davis and Oakes,[6] amounts to less than 3 parts in 1,000, and is affected but little by the pH or the character of the gelatin.

[6] Davis, C. E., and Oakes, E. T., *J. Am. Chem. Soc.*, 1922, xliv, 464.

The results of the viscosity measurements are represented in Fig. 3. In the more alkaline solutions the viscosity was slowly decreasing, the change in about 1 hour being represented in the upper curve by the distance between two points at the same pH. The lower curve represents a separate experiment made with a different lot of isoelectric gelatin. In each case the minimum viscosity was found to be

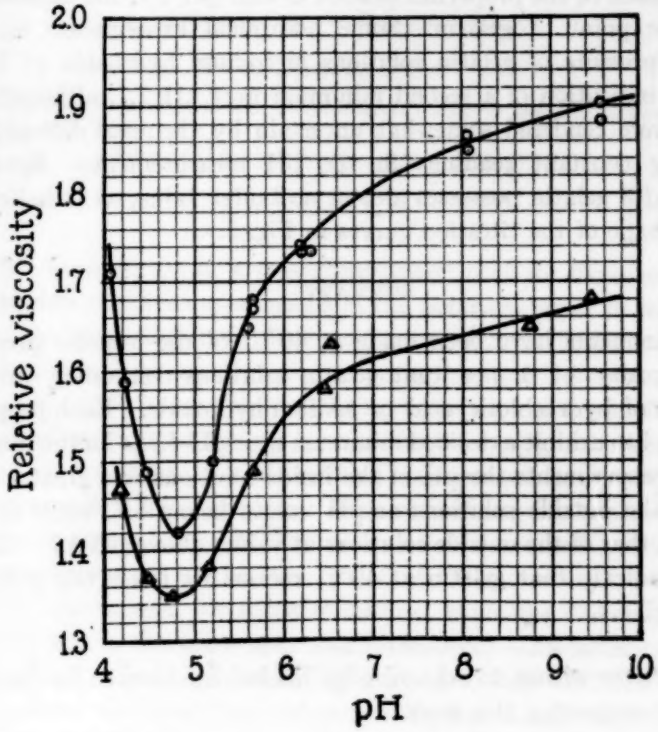

FIG. 3. Effect of pH on viscosity of 1 per cent gelatin solutions at 40°C.

near pH 4.7, and the curves are similar in shape to those obtained by Loeb[7] at 24°C. A curve of quite different shape was obtained by Davis and Oakes,[6] and reproduced by Wilson[8] in support of his idea that gelatin at 40° had an isoelectric point at pH 7.7. Possibly

[7] Loeb, J., *J. Gen. Physiol.*, 1920–21, iii, 104.

[8] Wilson, J. A., The chemistry of leather manufacture, New York, 1923, 113.

the different shape of their curve may be due to their method of preparing the solutions, which included heating the gelatin to 75°C.

While the present experiments do not exclude the possibility of additional secondary minima in osmotic pressure and viscosity, such as that observed by Wilson and Kern[1] in the case of swelling in phosphate buffers, they do indicate that at 40° the principal minimum in each of the properties studied is near pH 4.7, the established isoelectric point of gelatin. Some additional experiments on the osmotic pressure of gelatin solutions in sodium hydroxide at 24°C. gave no indication of a second minimum near pH 7.7, although the results were rendered somewhat uncertain by the great difficulty of obtaining constant readings in the pH measurements. Between pH 7 and 8 gelatin possesses almost no buffer value, as is indicated by the shape of the titration curves in Fig. 2.

SUMMARY.

Measurements have been made at 40°C. of the osmotic pressure and viscosity of 1 per cent gelatin solutions containing varying amounts of hydrochloric acid or sodium hydroxide. Each property was found to exhibit a decided minimum near pH 4.7. In the osmotic pressure experiments the pH of the inside solutions was greater than that of the outside solutions at pH values below 4.7, while it was less than that of the outside solutions at values above pH 4.7. These results indicate that gelatin at 40°C. retains its isoelectric point at about pH 4.7.

The writer wishes to acknowledge his indebtedness to Dr. Jacques Loeb for suggesting this work.

THEORY OF REGENERATION BASED ON MASS RELATION.*

III. Further Experiments on the Cause of the Polar Character of Regeneration.

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I.

It had been stated in a preceding paper[1] that we have to decide between two possible explanations of the polar character of regeneration in the stem of *Bryophyllum calycinum;* namely, first, Sachs' idea that the polar character of regeneration in the stem is due to a difference in the chemical constitution of the ascending and descending sap, the ascending sap containing special shoot-forming substances collecting at the apex, the descending sap containing root-forming substances collecting at the base. The second possible explanation is that the anlagen for roots and shoots are contained in different histological elements of the stem and that the ascending sap reaches primarily the anlagen for shoot formation, while the descending sap reaches primarily the anlagen for root formation. This latter explanation does not exclude the possibility that there exist chemical differences between the ascending and descending sap; it only assumes that the chemical differences between ascending and descending sap which in all likelihood exist are not the cause of the polar character of regeneration in the stem.

* In the preceding papers the term mass action instead of mass relation was used. Since the objection might be raised that the proportionality between the mass of leaf or stem and the mass of shoots or roots regenerated by them may not be entirely the result of mass action, it is safer to use the term mass relation for the present instead of mass action.

[1] Loeb, J., *J. Gen. Physiol.*, 1922–23, v, 831.

We have already given one fact which seems to speak in favor of the second explanation and we will add some further observations favoring this explanation.

The sap in the leaf contains all the substances required for the growth of both roots and shoots. This is no mere surmise, but is demonstrated by the fact that from each notch of an isolated leaf both roots and shoots can arise, and that the mass of shoots and roots produced varies in proportion with the mass of the leaf. This leaves no doubt that the same tissue sap can give rise to entirely different organs and that the cause of the difference must lie in differences in the nature of the cells from which the different organs arise. It will be shown in this paper that the shoots or roots produced in a sufficiently small piece of stem containing one or more large leaves originate almost entirely from the sap sent out by the leaf. If it is intended to explain the polar character of the regeneration in this case on the basis of a chemical difference of the sap sent out by the leaf to the two opposite poles of the stem, it will be first necessary to explain how the sap sent out by a leaf can be separated into two chemically distinct masses, one to travel in an ascending the other in a descending direction in the stem. The writer is not aware that such an assumption is justified on the basis of our present knowledge.

Slender stems with five nodes (designated as 0, 1, 2, 3, and 4) were cut out from plants almost but not quite 1 year old. All the leaves were removed except the pair of leaves in the third node (Fig. 1). The stems were split longitudinally between the two leaves, and one leaf was left in connection with its half stem (I, Fig. 1), while the other leaf was detached and suspended with its apex dipping into water (III, Fig. 1). The two half stems, I and II, dipped also with their base into water. Half stem I, with a leaf attached in Node 2, produced larger shoots at the apex than the other half of the stem II with no leaf. The difference in root formation was in the same sense and still greater. The experiment lasted from October 18 to November 9. Six half stems without leaves (II, Fig. 1) produced 28 mg. dry weight of shoots, while the other six half stems each with a leaf produced (during the same time under equal conditions) 237 mg. dry weight of shoots. Of this quantity about 209 mg. were, therefore, furnished by the leaves. The six isolated sister leaves (III, Fig. 1) produced 292 mg. of shoots.

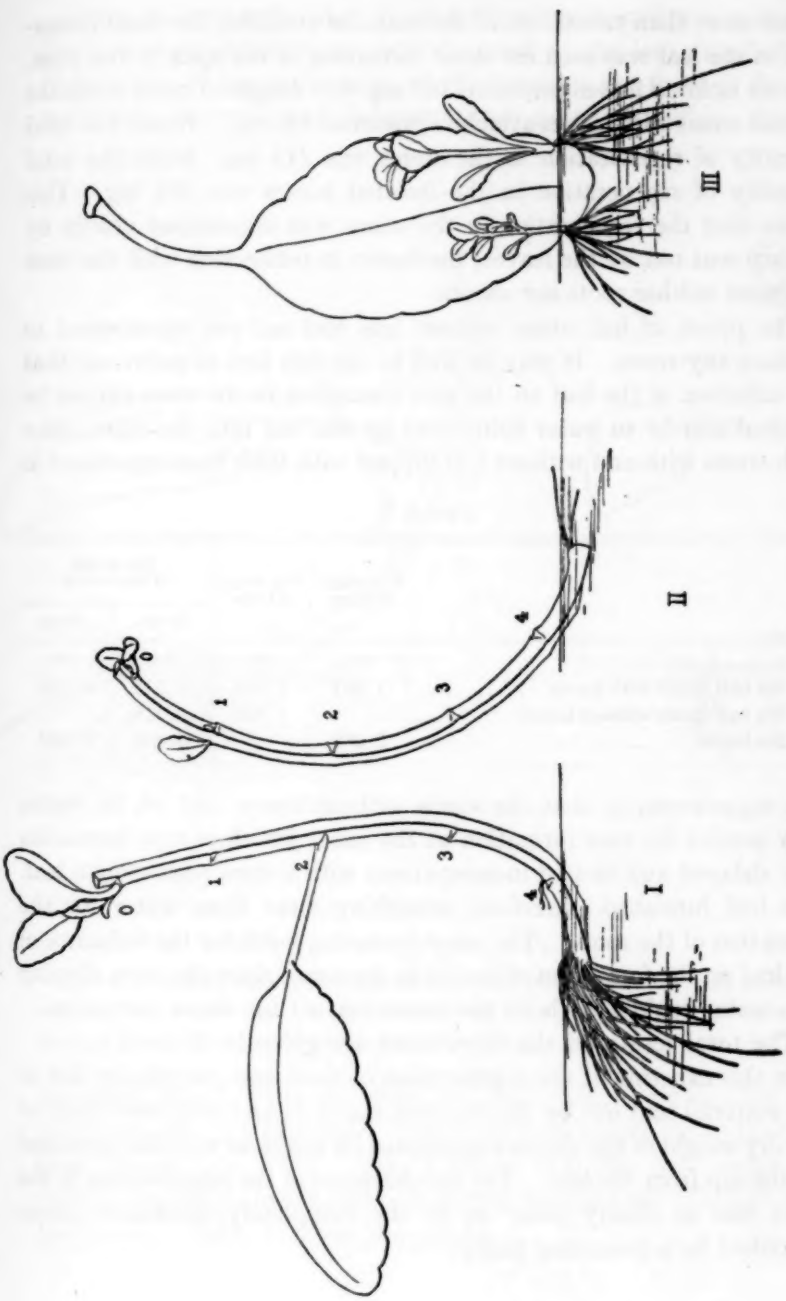

FIG. 1. Stems split longitudinally. Left half (I) with leaf in middle, produces a greater mass of shoots and roots than the right half (II) without a leaf. (October 18 to November 9, 1923.)

Hence more than two-thirds of the material available for shoot formation in the leaf was used for shoot formation in the apex of the stem. The six isolated leaves produced 102 mg. dry weight of roots while the six half stems with leaves attached produced 136 mg. Hence the total quantity of regeneration in the stems was 373 mg., while the total quantity of regeneration in the isolated leaves was 394 mg. This shows that the regeneration in the stems was determined chiefly by the sap sent out by the leaves; the leaves in connection with the stem produced neither roots nor shoots.

The pieces of half stem without leaf had not yet commenced to produce any roots. It may be well to use this fact to point out that the influence of the leaf on the root formation in the stem cannot be ascribed merely to water being sent by the leaf into the stem, since both stems with and without leaf dipped with their base into water in

TABLE I.

	Dry weight of leaves.	Dry weight of stems.	Dry weight of regenerated	
			Shoots.	Roots.
	gm.	gm.	gm.	gm.
I. Six half stems with leaves	1.993	1.889	0.237	0.136
II. Six half stems without leaves		1.836	0.028	
III. Six leaves	2.005		0.292	0.102

this experiment, so that the stems without leaves had all the water they needed for root formation at the base; yet their root formation was delayed and limited in comparison with a stem possessing a leaf. The leaf furnished, therefore, something more than water for the formation of the roots. The same reasoning holds for the influence of the leaf on the formation of shoots in the stem, since the stem dipping into water could absorb all the water needed for shoot formation.

The total results of the experiment are given in Table I.

In this experiment the regeneration of roots was completely due to the material sent out by the leaf into the stem and over two-thirds of the dry weight of the shoots regenerated by the stem was also furnished by the sap from the leaf. Yet the character of the regeneration in the stem was as clearly polar as in the completely defoliated stems described in a preceding paper.

It was to be expected that the quantity of regeneration in an isolated piece of stem should vary with the mass of the leaf attached. In eight stems, of five nodes each, all the leaves except those in the third node were removed and the stems were split lengthwise. One leaf

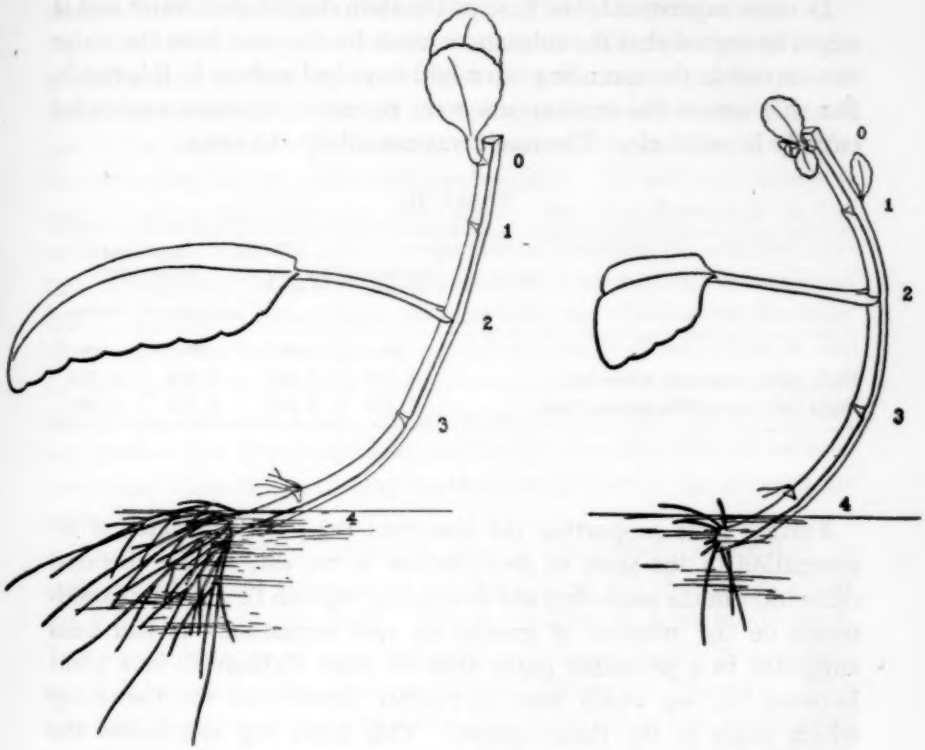

FIG. 2. Pieces of stem split longitudinally, left piece with whole leaf, right piece with leaf reduced in size. Mass of shoots and roots produced in stem varies with mass of leaf. (October 15 to November 7, 1923.)

remained intact, while the sister leaf was reduced by cutting away about two-thirds of the leaf. The stems were suspended so that their base dipped into water. Fig. 2 shows that the half stems with reduced leaves produce a correspondingly smaller mass of apical shoots and roots. Table II gives the results.

If account is taken of the fact that part of the material sent by the leaf into the stem is consumed for the growth of the latter (as was shown in preceding papers), the mass of shoots produced in the stem varies approximately with the mass of the leaf.

In these experiments the base of the stem dipped into water and it might be argued that the substances taken by the stem from the water and carried in the ascending sap might have had a share in this result. For this reason the experiments were repeated on stems suspended entirely in moist air. The result was essentially the same.

TABLE II.

	Dry weight of leaves.	Dry weight of stems.	Dry weight of regenerated	
			Shoots.	Roots.
	gm.	*gm.*	*gm.*	*gm.*
Eight half stems with whole leaf.....	3.550	3.318	0.404	0.218
Eight half stems with reduced leaf.....	1.273	3.503	0.201	0.046

II.

Further facts supporting the idea that the polar character of regeneration in the stem of *Bryophyllum* is not due to the chemical differences in the ascending and descending sap can be given by experiments on the influence of gravity on root formation. It had been suggested in a preceding paper that we must distinguish in a plant between the sap which flows in regular vessels and the tissue sap which exists in the tissue spaces.[2] This latter sap can follow the influence of gravity when the tissue spaces are sufficiently large; while the sap in the vessels cannot follow gravity at all or only to a negligible extent. We shall see a corroboration of this suggestion later in this paper. The fact that the tissue sap follows gravity gives us a chance to decide whether the polar character of regeneration in the stem is due to any chemical differences between ascending and descending sap or whether it is due to any difference in the nature of the tissues which these two kinds of sap reach primarily in the plant; the ascending sap reaching primarily the anlagen for shoots while the descending sap

[2] Loeb, J., *J. Gen. Physiol.*, 1919–20, ii, 373.

reaches primarily tissues which give rise to roots. We shall see that in a stem suspended horizontally the descending sap from a leaf collects in the lower side of such a stem giving rise to the formation of roots; and that this formation of roots in the under side of a stem increases with the mass of an apical leaf. If now the polar character of regeneration in a stem were due to a chemical difference between the ascending and descending sap from a leaf, such a root formation should not occur in the under side of the apical part of a stem which has a leaf at its base, since in this case the ascending sap should contain shoot-forming but no root-forming substances. We shall see, however, that a leaf at the base of a stem causes also root formation on the under side of the apical part of a stem placed horizontally and that this root formation is exclusively due to ascending sap sent out by the basal leaf. This leaves little doubt that the influence of a leaf on the polar character of the regeneration in a vertical stem of *Bryophyllum* is due not to any difference in the chemical constitution of the ascending and descending sap from a leaf, but is due to the fact that the descending sap reaches in a stem suspended vertically primarily anlagen for root formation while the ascending sap reaches primarily the anlagen for shoot formation.

When a completely defoliated stem is suspended horizontally in moist air, the polar character of regeneration is not changed as far as shoot formation is concerned. Shoots continue to form in the apex. The polar character of root formation undergoes, however, a change which becomes striking when not all the leaves are removed. It is true that roots continue to be formed at the extreme base of the stem and this root formation undergoes no change when the stem is put into a horizontal position. The change manifests itself in the fact, however, that in addition to this polar form of root formation there appears now a second form of root formation; namely, along the whole lower side of the stem. This latter effect is due to the collection of tissue sap on the lower side of the stem under the influence of gravity. This peculiar influence of gravity on the regeneration of horizontally placed stems finds its explanation in the assumption already referred to that there are two channels for the distribution of sap in the stem; first, by the vascular system, and second, by the gaps between cells and tissues. The sap flow in the vascular bundles is little if at all influenced by

gravity, and those forms of regeneration which depend upon the vascular sap are little if at all affected by gravity. We notice, therefore, that shoots continue to form in abundance at the apex and roots at the extreme base of a stem placed horizontally.

The tissue sap, however, follows gravity, collecting on the lower side of the stem where roots are formed but no shoots. In this respect a stem suspended horizontally differs from a leaf which when suspended horizontally forms both roots and shoots on the lower edges. This difference in the influence of gravity on regeneration in the leaf and the stem is a strong argument in favor of the tissue theory of polarity and against the hormone theory. For if it is true that the descending sap which is responsible for the root formation at the base of a stem has this effect because it reaches primarily root-forming tissue we should expect that the tissue sap collecting on the lower side of a horizontally placed stem should give rise only to roots but not to shoots, as it actually does.

Fig. 3. shows the regeneration which occurs in stems suspended horizontally in moist air. The upper stem is entirely defoliated. It has formed shoots at the apex and roots on the upper as well as on the lower side of the most basal, fifth node, but more on the lower side. There are also a few roots on the lower side in the fourth node and none on the upper. There is only a slight indication of the influence of gravity on the growth of roots. The geotropic curvature is only slight as it always is in completely defoliated stems.

The lower drawing in Fig. 3 gives the influence of a pair of *apical* leaves on the formation of roots in a stem which was suspended horizontally. Both the upper and lower stems in Fig. 3 were in the same aquarium and both experiments were carried out simultaneously (November 18 to December 13, 1923). At and near the base, the lower stem (with two leaves at the apex) forms roots on the upper side as well as on the lower. In Nodes 1, 2, and 3, however, and in the internode between 2 and 3, abundant roots are formed on the lower side exclusively. In order to prevent the stem from undergoing too much curvature and from being thrown altogether out of an approximately horizontal position, it was loosely tied to a piece of wood as shown in the drawing.

It can be shown that the mass of the roots formed in horizontally placed stems increases with the mass of the apical leaf. The two stems in Fig. 4 were suspended simultaneously, the lower stem with a whole leaf at the apex formed more roots than the upper stem with a leaf reduced in size (May 4 to June 5, 1923).

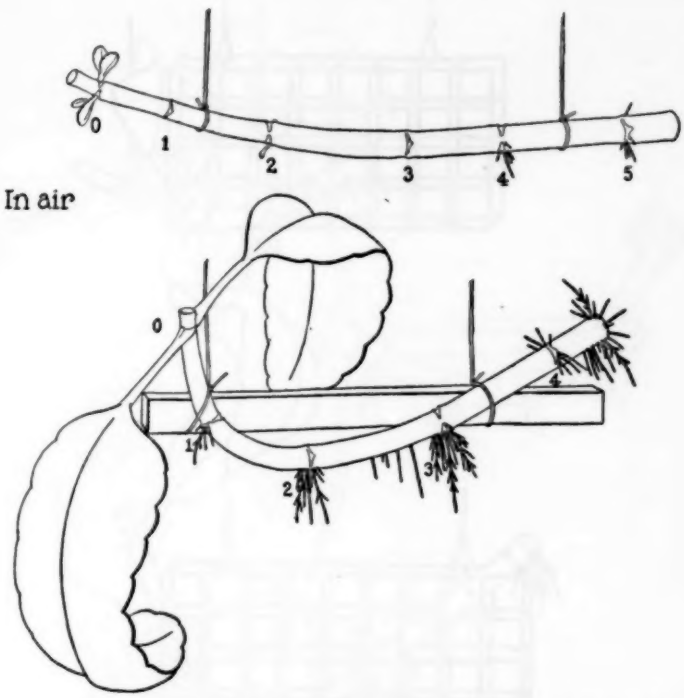

FIG. 3. Stems suspended horizontally form roots on the under side of the stem. Lower stem with two apical leaves forms more roots than upper stem without leaves. (November 18 to December 13, 1923.)

It had already been shown in a preceding paper that the mass of air roots formed in a stem suspended horizontally in moist air increases in proportion with the mass of the apical leaf, so that there is no doubt about the fact that the descending sap sent out by a leaf is responsible for the greater part of roots formed in a stem placed horizontally.

Now if the sap sent out by the apical leaf in the ascending direction is as capable of causing root formation as is the descending sap, we should be able to demonstrate that when a stem possessing a pair of leaves at the base is suspended horizontally, roots will also form on the

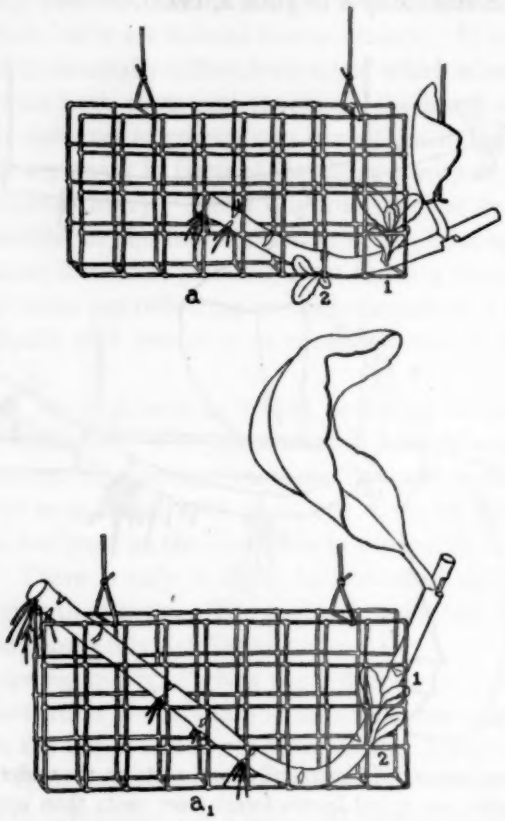

FIG. 4. a and a_1 were originally straight stems placed horizontally, a_1 with whole leaf, a with reduced leaf. Root formation on lower side in stem a_1 with whole leaf is greater than in stem a with reduced leaf. (May 4 to June 5, 1923.)

lower side of the stem apically from the leaf. That this is the case is shown in Fig. 5. The stem had a pair of large leaves at the base, one of which dipped into water. In order to prevent excessive geotropic curvature the stem was loosely fastened to a piece of wood. Nevertheless some geotropic curvature occurred. The stem has formed no

roots on the upper side but an abundant mass of roots on the lower side and some on the sides. The drawing was made on the 11th day of the experiment. Since the same stem without a leaf would in this time have formed practically no roots on the lower side the enormous root formation on the lower side of the apical stem must have been due

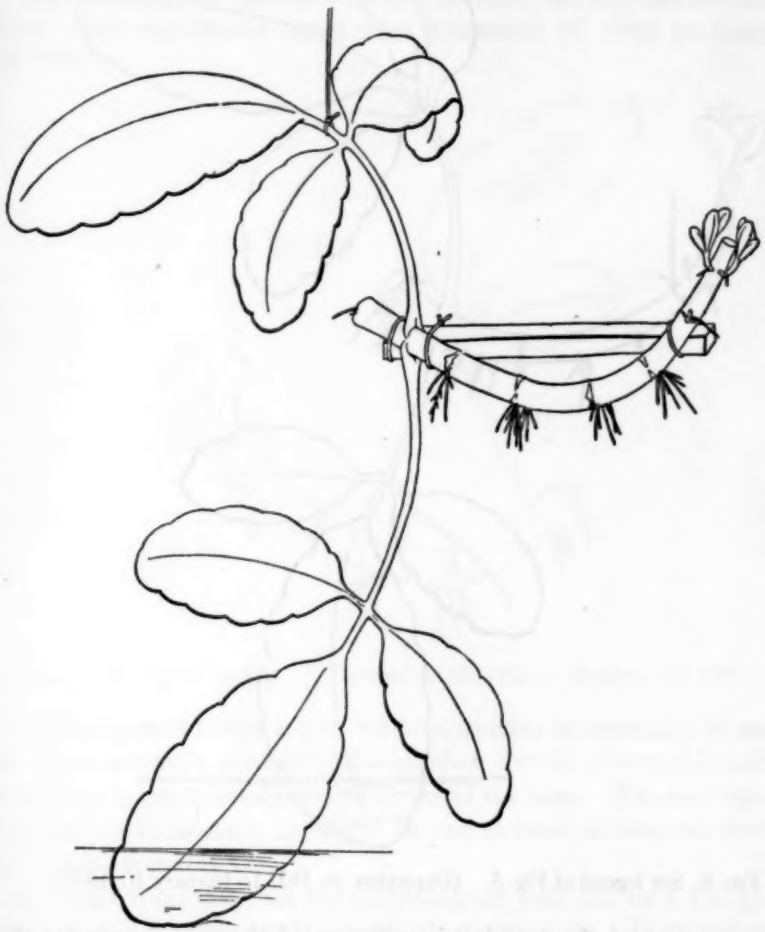

FIG. 5. Stem placed horizontally with two leaves at base. In the apical part of the stem roots are formed on the lower but not on the upper side, showing that the ascending sap from a leaf can form roots as well as the descending sap. (December 16 to December 27, 1923.)

to the ascending sap sent out by the basal leaves. The same results were obtained in a large number of experiments though the root formation varies in different stems, especially with the mass of the leaf. But the same is true for the root formation caused by the apical leaf.

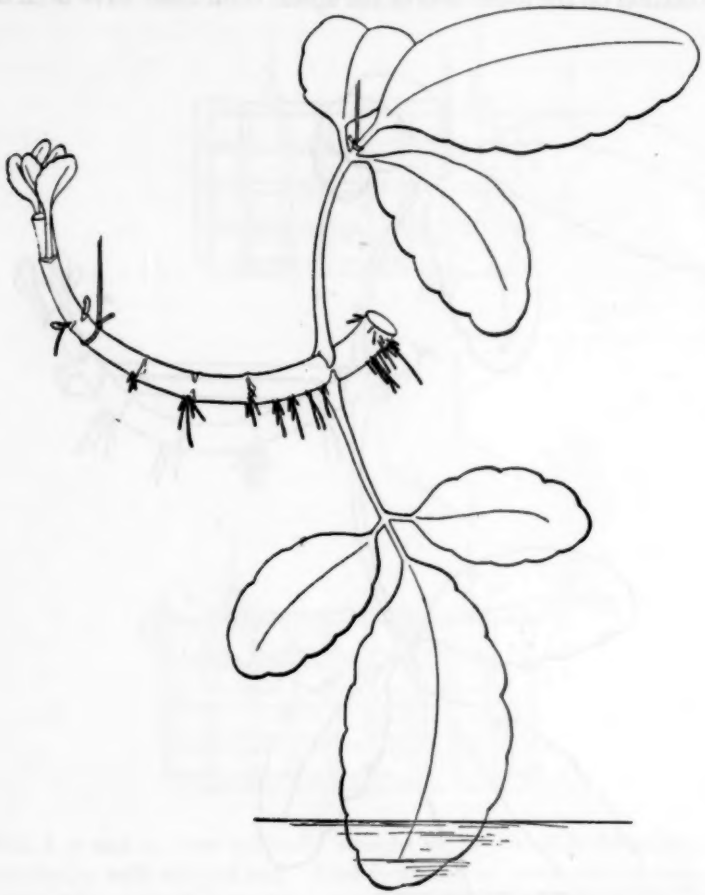

FIG. 6. See legend of Fig. 5. (December 16, 1923 to January 10, 1924.)

On account of the seeming importance of the experiment for the theory of polarity in regeneration, a few more results of a similar character may be described. Fig. 6 is a stem which was straight at the beginning and had been suspended horizontally. The stem had a

pair of large leaves at the base. Roots were formed on the lower side of the stem only, not only basally from the leaves but also apically. It is worthy of notice that some of the roots situated apically from the leaf developed in the internode. The experiment lasted from December 16, 1923 to January 10, 1924.

Fig. 7 is a similar experiment where the stem had only one leaf at the base. The experiment lasted from December 17, 1923 to January 10, 1924.

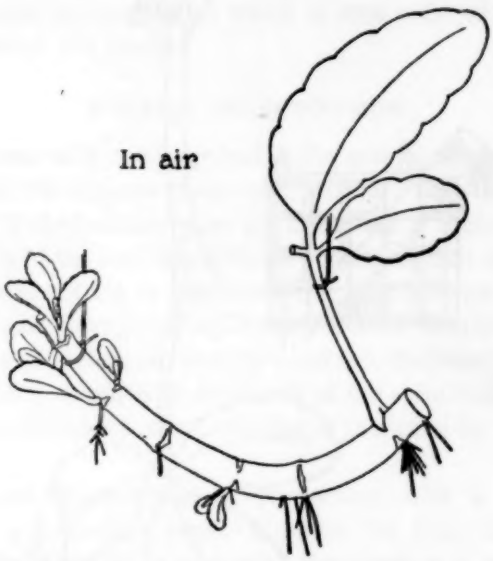

FIG. 7. See legend in Fig. 5. (December 17, 1923 to January 10, 1924.)

While a stem without a leaf, when suspended horizontally in moist air, forms few roots, a considerable number of roots is formed in such a stem when it contains one or two leaves at the base. The root formation must in these cases, therefore, be due to material sent out by the leaf in the ascending sap.

It follows from this that the ascending sap sent out by a leaf gives rise to roots if it reaches cells capable of forming roots. If, therefore, a stem suspended vertically gives rise only to shoots it must be due to the fact that in a stem suspended vertically the ascending sap does not collect primarily in the cells where roots can be formed, but collects primarily near the most apical anlagen for shoots.

In all these cases the stems in which the roots formed had bent geotropically the under sides of the stem where the roots grew being convex. It might be argued that this convex form was the cause of the root formation. This is refuted by the fact already published years ago, that if a stem is bent passively and suspended so that the convex side of the stem is above, the roots form on the lower concave side of the stem.[3]

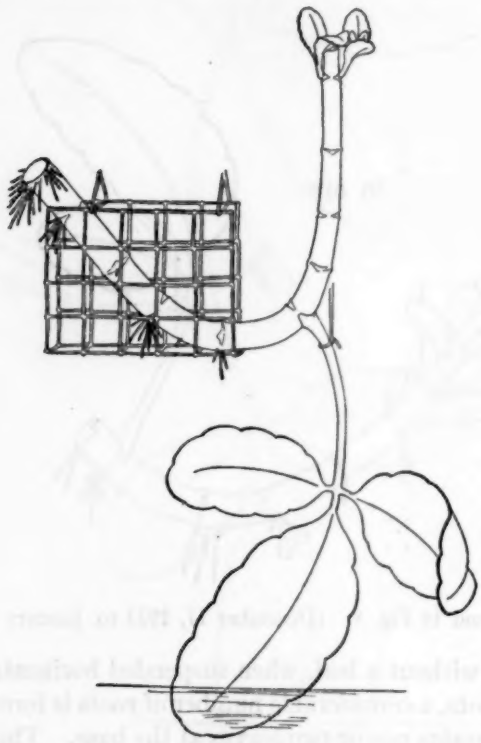

FIG. 8. Leaf in middle of stem. Root formation and geotropic curvature basally from the leaf. (December 23, 1923 to January 10, 1924.)

When the leaf is in the middle of a stem originally suspended horizontally the roots form chiefly in that part of the stem which is situated basally from the leaf, for the reason that only this part remains in a sufficient horizontal position, the part of the stem situated

[3] Loeb, J., *Bot. Gaz.*, 1917, lxiii, 25.

apically from the leaf assuming a vertical position on account of the geotropic curvature of the stem (Fig. 8).

A few words may be said in regard to the geotropic curvature. When a piece of stem suspended horizontally has one or two leaves at the base (as in Figs. 5, 6, and 7), geotropic curvature takes place in the stem apically from the leaf; when the leaf is in the middle chiefly basally from the leaf. This statement must replace the statement made in a preceding paper that the piece of stem apically from the leaf cannot bend geotropically; which is true only when the leaf is in the middle of the stem.

SUMMARY AND CONCLUSION.

1. Small stems with one large leaf in the middle produce shoots and roots chiefly at the expense of material sent into the stem by the leaf. Since the sap of the leaf can cause the formation of roots and shoots in the same notch of the leaf, it is difficult to assume that the polar character of the regeneration in the stem is due to a chemical difference in the ascending and descending sap sent by the leaf into the stem.

2. When the ascending sap sent by a leaf into the stem is caused with the aid of gravity to reach those tissues of the stem from which roots are formed, an abundant root formation is produced by the ascending sap.

3. These two observations in connection with a fact already published in a preceding paper[1] support the idea that the polar character of regeneration in a stem of *Bryophyllum* is not due to any of the chemical differences between the ascending and descending sap but to a difference in the nature of the tissues or anlagen which are primarily reached by the ascending and descending sap.

THE ULTIMATE UNITS IN PROTEIN SOLUTIONS AND THE CHANGES WHICH ACCOMPANY THE PROCESS OF SOLUTION OF PROTEINS.

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I.

A number of facts suggest that the genuine proteins are kept in aqueous solution by the forces of attraction between the molecules of proteins or their polar groups and the molecules of water, or, in other words, that they form true solutions. Such facts are the high concentrations of salts required for precipitation of genuine proteins (regardless of valency of ion), the increase in solubility of isoelectric gelatin by salts, increasing with the valency of either ion of the salt,[1] the applicability of the principle of constancy of solubility product,[2] and others. All this, however, does not give us any insight into the nature of the ultimate units in these solutions; namely, whether the ultimate unit is the individual protein molecule or a small or a large aggregate of molecules; since it is quite conceivable that molecules of proteins, though dragged into the water by their polar groups form aggregates through the forces of cohesion between their non-polar groups. Such a possibility is strongly suggested in the case of proteins which set to a gel, *e.g.* gelatin.

It is possible to arrive at some conclusion concerning the molecular nature of the ultimate particles in protein solutions through measurements of viscosity. When finely powdered particles of solid isoelectric gelatin are put into a solution of an acid, the particles swell. The cause of this swelling is revealed when the concentration of the H ions and

[1] Loeb, J., *Arch. néerl. physiol. homme et animaux*, 1922, vii, 510; *J. Gen. Physiol.*, 1922–23, v, 479.

[2] Cohn, E. J., and Hendry, J. L., *J. Gen. Physiol.*, 1922–23, v, 521.

the anions inside the particles and in the supernatant water are compared. It is found that the molar concentration of the two oppositely charged ions is different inside the gelatin granules from what it is outside, and the ratio of the two kinds of oppositely charged monovalent ions of the acid is expressed by the following equation.

$$\frac{\text{concentration of hydrogen ions inside}}{\text{concentration of hydrogen ions outside}} = \frac{\text{concentration of anions outside}}{\text{concentration of anions inside}}$$

or

$$\log \frac{[H^+] \text{ inside}}{[H^+] \text{ outside}} = \log \frac{[Cl^-] \text{ outside}}{[Cl^-] \text{ inside}}$$

or

$$\text{pH inside minus pH outside} = \text{pCl outside minus pCl inside.}$$

This was demonstrated in the following way. 1.0 gm. of finely powdered dry isoelectric gelatin granules was put into 100 cc. H_2O containing different quantities of 0.1 N HCl; namely, 8, 10, 12, 16, and 20 cc. The suspension was allowed to stand for 1 hour at 20°C. The outside solution was then filtered off and its pH was determined electrometrically. The granules of gelatin were melted and the pH of the liquid was determined in the same way. The pCl outside was, of course, equal to the pH outside. Knowing the volume of the filtrate the quantity of Cl in the filtrate could be calculated and by deducting this value from the value of HCl originally added, it was possible to calculate the Cl held by the gelatin. (It had been shown already that this Cl exists in the form of Cl ions.) In this way the concentration of chlorine inside was obtained. The $\log \frac{[Cl^-] \text{ outside}}{[Cl^-] \text{ inside}}$ could now be calculated (Table I).

Considering the limits of accuracy the agreement in Table I between the values of pH inside − pH outside and pCl outside − pCl inside is satisfactory.

This distribution of the H and Cl ions between the micelles of gelatin and the supernatant water is characteristic for membrane equilibria and finds its explanation by Donnan's theory of membrane equilibria, which predicts this striking distribution if one ion cannot

diffuse through a membrane or a gel which is readily permeable to the ordinary ions of electrolytes. The non-permeable ion is in this case the protein ion which in the powdered particle or micelle is held by the force of cohesion between the molecules (or rather the non-polar "oily" groups of the molecule) of gelatin.

As was first shown by Procter and Wilson, the unequal distribution of diffusible ions inside and outside the gel leads to an excess of total molar concentration of the diffusible ions inside the gel over that outside, and this excess increases the osmotic pressure inside over that in the outside solution. The consequence is the diffusion of more water into the gel and an increase in the volume of the solid particles.[3]

It had been shown that the swelling of the solid particles of gelatin and casein in acid leads to an increase in the viscosity of suspensions of these particles and that the influence of acid on the viscosity of

TABLE I.

Distribution of Diffusible Ions between Micelles of Gelatin and Surrounding Water at Osmotic Equilibrium. 1 Hour at 20°C.

Cc. 0.1 N HCl in 100 cc. solution	8	10	12	16	20
pH inside	3.14	2.91	2.70	2.37	2.17
pH outside	2.56	2.35	2.23	2.05	1.89
pH inside – pH outside	0.58	0.56	0.47	0.32	0.28
pCl outside – pCl inside	0.58	0.46	0.42	0.37	0.30

suspensions of powdered particles of originally isoelectric gelatin in water runs parallel to the influence of acid on swelling.[4] This parallelism is easily understood on the basis of Einstein's formula connecting the volume of particles dissolved or suspended in water with the viscosity of the solution or suspension.

According to Einstein, the viscosity of aqueous solutions is a linear function of the relative volume occupied by the solute in the solution,

$$\eta = \eta_0(1 + 2.5\varphi_0)$$

in which η_0 is the viscosity of the water at the temperature of the experiment, η the viscosity of the solution, and φ the fraction of the volume of the solution occupied by the solute. This formula can be

[3] Procter, H. R., and Wilson, J. A., *J. Chem. Soc.*, 1916, cix, 307.

used only when φ is very small and when the particles of the solute are spherical and large in comparison with the molecules of the solvent.

When powdered particles of gelatin swell in acid, water enters the particles and the value of φ in Einstein's equation increases. The main fact was that under the influence of acid the value of φ varies in the case of suspensions of powdered particles of gelatin or casein in acid according to Donnan's equation.

It was further shown that the viscosity of *solutions* of gelatin varies with the pH of the gelatin solution in exactly the same way as does the viscosity of *suspensions of powdered particles* of gelatin, and the only difference was that the viscosity of *freshly prepared solutions* of gelatin was always smaller than that of the *suspension of powdered particles* for the same concentration of originally isoelectric gelatin, the same pH of the gelatin, and the same temperature. The inference was that the influence of acid on the viscosity of gelatin *solutions* was also due to the swelling of micelles which in the solution of gelatin were, however, not visible; but from the fact that the freshly prepared solution of gelatin had a lower viscosity than the powdered gelatin and that the influence of acid on viscosity was also smaller in the solution than in the case of the powdered gelatin, it was to be inferred that when a solid particle of gelatin goes into solution, the result is a formation of units of different size, some units being possibly individual molecules or aggregates of so few molecules that the aggregates were too small to give rise to a Donnan equilibrium, while others were units consisting of enough molecules to give rise to a Donnan equilibrium and to undergo acid swelling.[4]

This inference was supported by the fact that solutions of genuine crystalline egg albumin which do not set to a gel (unless too much acid is added or unless the temperature is too high) have a very low viscosity. The addition of acid to solutions of genuine crystalline egg albumin causes only a slight increase in the viscosity of the solutions, while the same change in pH causes a considerable increase in the viscosity of solutions of gelatin.[4]

[4] Loeb, J., Proteins and the theory of colloidal behavior, New York and London, 1922.

Hedestrand[5] has shown that acid causes a slight increase in the viscosity of solutions of amino acids of a high concentration. This increase may have been due to the possible attachment of some water molecules to the ions of the amino acids (hydration). The amino acids form true solutions and it is not probable that they form aggregates capable of giving rise to a Donnan equilibrium. The low order of magnitude of the influence of acid on the viscosity of solutions of genuine crystalline egg albumin suggests that the influence of acid is either not due at all to swelling of micelles, but, perhaps, only or chiefly to a hydration of the individual molecules; or, if it is due to swelling, that there are only few aggregates capable of swelling in albumin solutions, since it can be shown that wherever there are particles capable of acid swelling present in a solution, the influence of acid on the viscosity is of a very high order.

It seems, therefore, as if the changes in viscosity which accompany the process of solution of solid particles of protein in acid might give us some information concerning the ultimate units of protein which are formed in the process of solution.

II.

The Viscosity of Solutions of Genuine and of Suspensions of Denatured Crystalline Egg Albumin at 24°C.

1 gm. of dry, finely powdered isoelectric egg albumin, denatured by heating, going through a sieve with mesh 200, was stirred up in 100 cc. H_2O containing 10 cc. of 0.1 N HCl and the time of outflow was measured at constant temperature of 24°C. at stated intervals (Table II) through a straight viscometer.

Table II gives also the relative viscosity of a 1 per cent solution of originally isoelectric genuine crystalline egg albumin, also in 100 cc. 0.01 N HCl. The viscosity was in both cases of a very low order of magnitude and did not change.

The influence of different concentrations of acid was almost negligible for both forms of albumin, denatured and genuine. 1 gm. of originally isoelectric albumin was put into 100 cc. water containing

[5] Hedestrand, G., *Z. anorg. u. allg. Chem.*, 1922, cxxiv, 153.

varying quantities of 0.1 N HCl. The viscosity was measured after the albumin had been in the acid solution for 1 hour at 24°C. (Table III).

TABLE II.

Viscosity at 24°C. of 1 Gm. Albumin in 100 Cc. of 0.01 N HCl.

Time after mixing.	Relative viscosity.	
	1 per cent suspension of denatured egg albumin.	1 per cent solution of genuine egg albumin.
min.		
5	1.02	1.04
10	1.02	1.05
15	1.02	1.04
25	1.02	1.04
35	1.02	1.04
45	1.04	1.04
55	1.03	1.04
65	1.04	1.05
75	1.03	1.04

TABLE III.

Relative Viscosity of 1 Gm. Albumin in 100 Cc. HCl Solutions after 1 Hour.

0.1 N HCl in 100 cc. H_2O.	1 per cent suspension of denatured egg albumin.	1 per cent solution of genuine egg albumin.
cc.		
0	1.03	1.03
2	1.01	1.04
4	1.01	1.04
8	1.01	1.05
12	1.01	1.05
16	1.00	1.05
20	1.00	1.04
30	1.00	1.04
40	1.01	1.04
50	1.01	1.03

It is obvious that there is no measurable effect of the acid on the viscosity of suspensions of finely powdered particles of denatured albumin; there is a slight but unmistakable effect on genuine egg albumin, the viscosity rising from 1.03 to 1.05 when acid is added and falling again to 1.03 when more acid is added.

Similar results are obtained with suspensions of insoluble casein particles when 1 gm. of very finely divided isoelectric casein (going through mesh 200) is put into 100 cc. of water containing acids which form insoluble casein salts—such as trichloroacetic, sulfuric, and sulfosalicylic. The viscosity of the casein remains very low since no swelling can occur. This is obvious from Table IV.

Similar results were obtained with sulfuric and sulfosalicylic acid.

All these experiments show that where there are no particles capable of giving rise to a Donnan equilibrium and to swelling in acid, the order of magnitude of the viscosity is low and the effect of acid on the viscosity is zero or very small.

TABLE IV.

Relative Viscosity of a Suspension of 1 Per Cent Casein after 1 Hour in Various Concentrations of Trichloroacetic Acid, at 24°C.

0.1 N trichloroacetic acid in 100 cc. H_2O.	Relative viscosity of casein.
cc.	
0	1.02
2	1.02
4	1.04
8	1.08 (?)
12	1.02
16	1.04
20	1.03
30	1.02
40	1.02
50	1.02

Hence we come to the conclusion that a low order of viscosity in a protein solution in the presence of acid indicates that the solution contains few or no micelles capable of swelling.

III.

Changes in Viscosity Accompanying the Solution of the Sparingly Soluble Type of Casein.

Entirely different results are observed when finely powdered isoelectric casein is put into solutions of an acid such as HCl or H_3PO_4, in which the casein is soluble. In this case the solution of the micelles

is preceded by swelling and the swelling varies with the concentration of acid and with the addition of neutral salts in agreement with the theory of membrane equilibria. It had also been shown in a book and in a preceding paper[6] that the viscosity of the casein chloride or phosphate solution varies with the relative volume of the casein particle in the solution, and that the comparatively high viscosity of casein solutions in acid is due to the swelling of micelles.

Two processes go on simultaneously in such casein solutions which affect the viscosity of the solution in an opposite sense, first, the swelling of the micelles under the influence of the acid by which the viscosity increases (since the value of φ in Einstein's equation increases with the swelling), and second, the solution of the micelles whereby the micelles are divided into units too small to give rise to a Donnan equilibrium.

In the older experiments on viscosity referred to, a casein preparation was used which was sparingly soluble. In this sparingly soluble form of casein, the power of cohesion between the molecules of casein was so great that a considerable hydrostatic pressure of the water forced into the micelles by the excess of osmotic pressure due to the Donnan equilibrium was required to smash the solid particles of casein, as a preliminary to their solution.[6] As a consequence the solution of the micelles became a function of the swelling inasmuch as solution of the micelles became possible only when the swelling reached a certain limit.

It is, however, possible to prepare a form of casein which is much more readily soluble in solutions of HCl so that the solution either ceases to depend upon the swelling of the micelles, or depends to a much smaller degree upon the swelling. We may assume that in this latter form of casein the molecules are held together with a smaller force of cohesion than in the case of the more difficultly soluble form of casein. The difference between the two forms lies in the preparation of the isoelectric casein from skimmed milk. To produce the less soluble casein, 1 N acid must be added very rapidly to the skimmed milk with moderate stirring. In this case some of the casein is perhaps

[6] Loeb, J., and Loeb, R. F., *J. Gen. Physiol.*, 1921–22, iv, 187. Loeb, J., Proteins and the theory of colloidal behavior, New York and London, 1922, 243.

"denatured." To produce the more rapidly soluble casein, 0.1 N acid must be added very slowly (running in through a capillary tube) with very energetic stirring. This latter type of casein can dissolve without undergoing any swelling, though as a matter of fact if the proper concentration of acid is selected, it can be shown that the actual solution may be preceded by a swelling. We will call the two types of casein the "less soluble" and the "more soluble," respectively.

It seemed of interest to compare the changes in viscosity which accompany the solution of the two types of casein in solutions of HCl. Since it is also possible to measure the change in size of an individual micelle under the microscope, and furthermore to measure the quantity of non-dissolved material, an exact method exists to test once more the conclusion arrived at in earlier publications that the changes in viscosity of the casein solution under the influence of acid are primarily and chiefly caused by the swelling of the casein micelles.

We will first discuss experiments on the "less soluble" casein.

Dry isoelectric casein was sifted through a sieve No. 200 and only the very fine granules which passed through this sieve were used. 1 gm. of such sifted powdered casein was put into 4, 8, 15, and 50 cc. of 0.1 N HCl to which enough water was added to make the volume 100 cc. Measurements of the rate of outflow were then made in stated intervals in which a straight form of a viscometer tube was used as described in previous publications. The temperature was always 24°C.

The curves in Fig. 1 represent the variations in the viscosity of 1 gm. of originally isoelectric casein during the first 3 hours in 100 cc. aqueous solution containing respectively 4, 8, 15, and 50 cc. of 0.1 N HCl. In 4 cc. of 0.1 N HCl the casein micelles swell slowly and the relative viscosity of the solution or suspension[7] increases slowly in 3 hours from 1.02 to 1.55. Later some of the casein goes into solution and the viscosity drops a little.

In 8 cc. of 0.1 N HCl the viscosity rises in 20 minutes from about 1.02 to 1.7. Then the process of solution proceeds more rapidly than the process of swelling and the viscosity drops rapidly to about 1.22. On

[7] By relative viscosity is meant the ratio of time of outflow of solution from viscometer over the time of outflow of pure water.

account of the Donnan equilibrium, the maximal swelling reached in 15 cc. of 0.1 N HCl is less than in 8 cc. HCl and hence the maximal viscosity is lower in 15 than in 8 cc. HCl. The solution proceeds at least as rapidly in 15 cc. as in 8 cc. and the viscosity drops parallel with that in 8 cc.

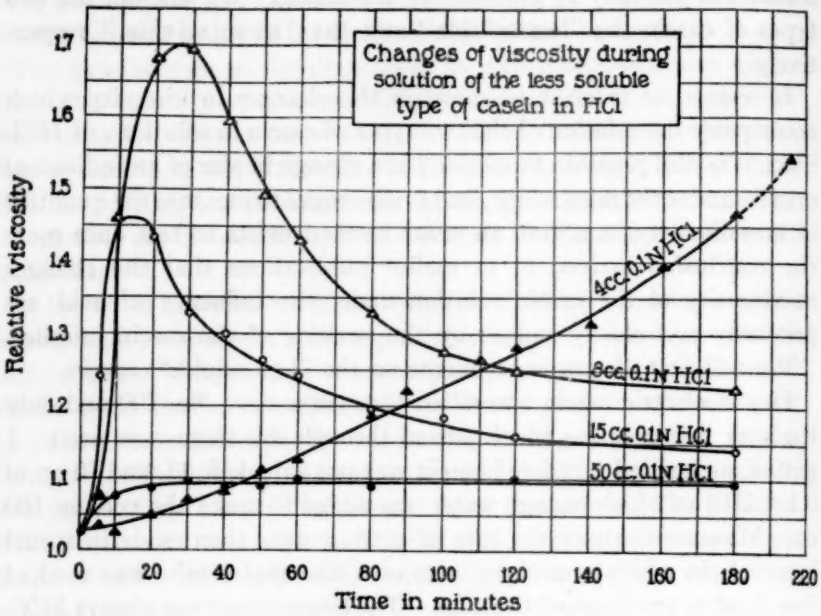

FIG. 1. Changes of relative viscosity of 1 gm. of powdered, nearly isoelectric casein of the less soluble type when put into 100 cc. H_2O containing 4, 8, 15, and 50 cc. of 0.1 N HCl. (The pH of these solutions after the casein was added was about 3.15, 2.45, 1.9, and 1.4, respectively.) Abscissæ, time after the powdered casein was put into the HCl solution; ordinates, relative viscosity. The viscosity rises at first on account of swelling of the particles and falls later on account of solution.

The Donnan equilibrium demands that the swelling should be considerably less in 50 than in 8 or 15 cc. 0.1 N HCl, and accordingly the viscosity rises but little in 50 cc. HCl, namely only to 1.1. On account of the small amount of swelling but little of this type of casein goes into solution in this concentration of HCl. Hence the viscosity remains almost unaltered at about 1.1 during the next 3 hours.

The application of the Donnan equilibrium to the explanation of swelling of the micelles demands that the addition of neutral salts to acid should diminish the swelling of the micelles, and hence should diminish also the viscosity; but since, for this difficultly soluble type of casein, solution depends upon the swelling reaching a certain quantity, the addition of salt should not only influence the viscosity (*i.e.* the ascending branch of the time curve) but also the descending part

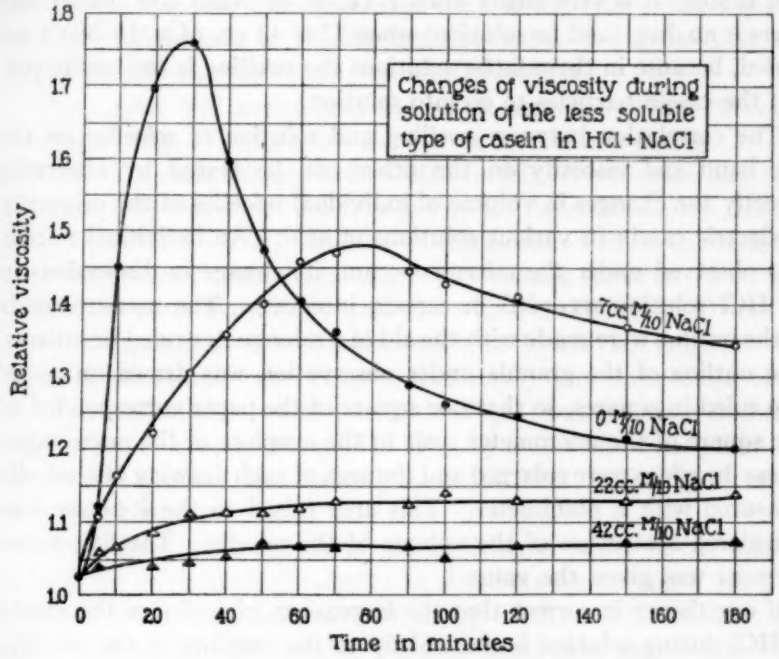

FIG. 2. Showing that the addition of NaCl depresses the viscosity.

due to solution of the casein. It is therefore possible to predict the effect of the addition of salt on the viscosity curves and the prediction is fulfilled. Fig. 2 gives the results of one series of experiments. The changes of viscosity with time were measured in the following four solutions.

8 cc. of 0.1 N HCl + 92 cc. H_2O.
8 " " " + 7 " M/10 NaCl + 85 cc. H_2O.
8 " " " + 22 " " " + 70 " "
8 " " " + 42 " " " + 50 " "

The theory of the Donnan equilibrium demands that the maximal swelling and hence the maximal viscosity should diminish with increasing concentration of salt, which is the case. While without NaCl the viscosity reaches a maximum of 1.76, the maximal viscosity is 1.46 in 7 cc. M/10 NaCl, 1.12 in 22 cc., and 1.06 in 42 cc. M/10 NaCl.

Furthermore, while the drop of the viscosity in the solution without salt is steep it is very slight when 7 cc. M/10 NaCl are added and there is no drop (and no solution) when 22 or 42 cc. of M/10 NaCl are added, because in these latter solutions the swelling is too low to permit the casein particles to go into solution.

The correlation between swelling and solution of micelles on the one hand and viscosity on the other can be tested by observing directly the changes in volume of individual micelles of the originally isoelectric casein in various solutions of acid. An individual micelle was observed under the microscope and its change in dimensions in an HCl solution recorded in certain intervals. The measurements of the outline were made with the aid of a micrometer ruled in squares. The outline of the granule under observation was drawn on paper also ruled in squares, so that one square of the paper corresponded to one square of the micrometer scale in the eyepiece of the microscope. These drawings were enlarged and the area of each drawing of a micelle measured with a planimeter. This area raised to the $\frac{3}{2}$ power was considered a measure of the volume of the micelle. The first measurement was given the value 1.

If our theory is correct that the increase in viscosity of the casein in HCl during solution is due chiefly to the swelling of the micelles and that the decline of the viscosity curves with time (Figs. 1 and 2) is due to solution, *i.e.* the transformation of parts of the micelles into units too small to give rise to a Donnan equilibrium, it must be possible to show that if we plot the values for the volume of the micelles as ordinates over the time, we must get a system of curves rising in time in a way similar to the rise in the curves in Fig. 1 or 2. The difference to be expected was in the following points. The solutions in which the viscosities were measured were frequently stirred, while the micelles used for measurements were not stirred since the same micelle had to be observed continuously. Hence solution commenced

earlier in the stirred solutions than in the non-stirred individual micelles. Furthermore, only a few individual micelles were observed in the direct volume measurements under the microscope and hence these values are afflicted with the error of individual variation. A third difference is the pH. If we make allowance for these facts and in the measurements of the area of the drawings of the micelles, we find that the changes in volume of the micelles actually observed in solutions of HCl have the same drift as the changes in viscosity. Fig. 3 gives the time as abscissæ and the change in volume of an individual micelle in each of the four solutions of HCl.

The reader will notice that the rising branches of the curves for viscosity (Fig. 1) agree with the curves for volume in Fig. 3. The volume rises most sharply in 8 cc. HCl (in Fig. 3) corresponding to an equally sharp rise of the viscosity in Fig. 1. After 30 minutes, the volume curve in 8 cc. HCl had to be discontinued since the surface of the micelle broke into fragments. The volume curve in 16 cc. HCl (Fig. 3) is lower than the volume curve for 8 cc. HCl (Fig. 3), and this also corresponds to the viscosity curve in 16 cc. HCl (Fig. 1). In 4 cc. HCl the volume curve (Fig. 3) rises slowly and no solution occurs and the viscosity curve in 4 cc. HCl (Fig. 1) has the same character. The volume curve in 50 cc. HCl (Fig. 3) is low and runs parallel to the axis of abscissæ, the viscosity curve for 50 cc. HCl (Fig. 1) having the same character.

Fig. 4 gives the influence of the addition of NaCl on the increase in volume of individual micelles in solutions containing 8 cc. 0.1 N HCl per 100 cc. solution, the volume curve in 8 cc. HCl (without salt) rises more rapidly and reaches a higher value than the volume curve for 7 or 22 cc. NaCl (Fig. 4) corresponding to the viscosity curves for the same salt values in Fig. 2. The character of the volume curve for 22 cc. NaCl in Fig. 4 agrees also with the character of the viscosity for 22 cc. NaCl in Fig. 2.

We can therefore say that the increase in viscosity which accompanies the first stage of the solution of powdered particles of the less soluble type of casein is due to the increase in the volume of the individual micelles of this type of casein.

Experiments were also made to ascertain the changes in viscosity of this type of casein in alkali where it is more readily soluble. Former

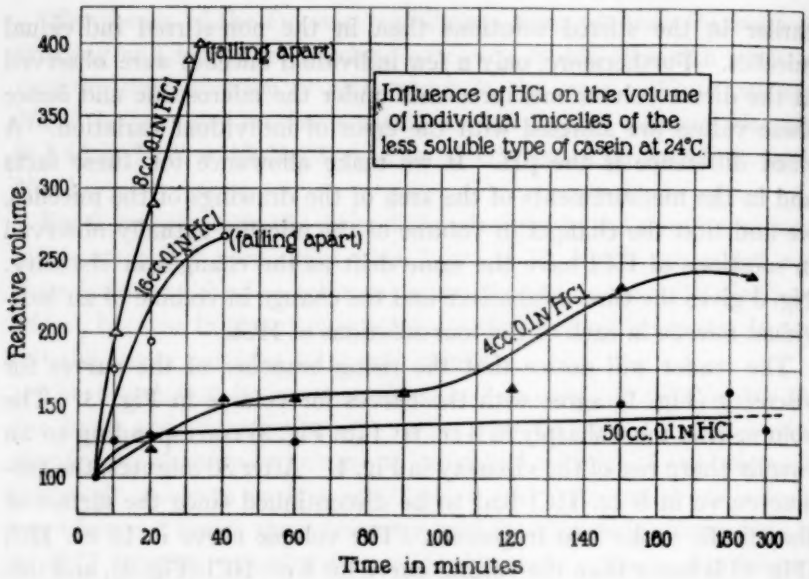

FIG. 3. Showing that the rise in viscosity in Fig. 1 was due to swelling of the casein particles. Abscissæ, time; ordinates, volume of individual granules. The curves for changes in volume of individual particles in Fig. 3 are similar to the curves for changes in viscosity, Fig. 1.

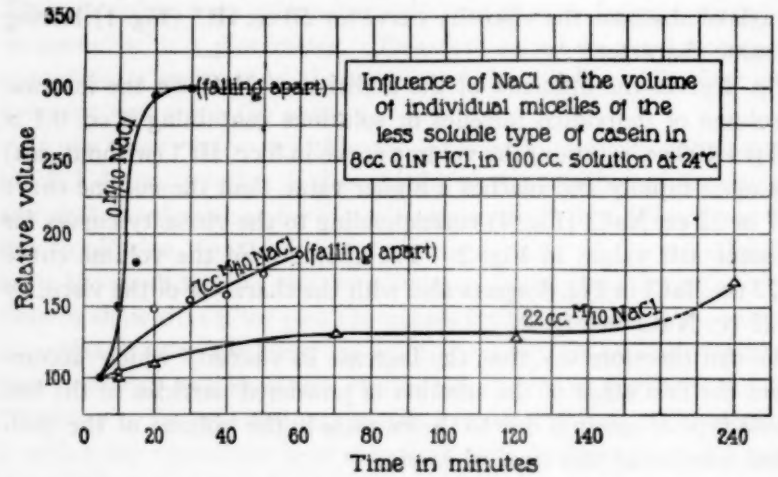

FIG. 4. Showing that the rise in viscosity in Fig. 2 was due to swelling of casein particles.

observations have shown that when powdered particles of isoelectric casein are put into alkali no swelling occurs but that the solution occurs in a different way. It was found that the viscosity of 1 gm. of originally isoelectric casein of this type rises in one minute to 1.11, reaching a maximum in 20 minutes of 1.16, and dropping again in 4 hours to 1.13. This indicates that the process of solution of this type of casein in alkali is accompanied by only an insignificant increase of viscosity and the low order of magnitude of viscosity indicates that the majority of units into which the casein dissolves in alkali are too small to undergo swelling.

IV.

Changes in Viscosity Accompanying the Solution of the More Soluble Type of Casein.

The swelling of the micelles of the more soluble type of casein in solutions of HCl should depend in a similar way upon the concentration of HCl and of salts as does that of the less soluble type discussed in the preceding chapter. The two types differ in regard to the solubility which shows itself in the fact that the micelles of the more soluble type of casein can go into solution even in the presence of higher concentration of acids or salts where the swelling is diminished in agreement with the Donnan equilibrium. In other words, the smashing of the micelle by the hydrostatic pressure of the water due to excess osmotic pressure inside the micelle is not a necessary prerequisite for the solution of this type of casein. Since this powder is not so readily wetted by the solution, the dry powder was ground up in a mortar with a trace of the acid solution, so as to insure its wetting and was then put into the solution of acid. Fig. 5 represents the changes in viscosity of 1 gm. of the more soluble type of casein in different concentrations of HCl. The size of the particles was the same as that used in the case of the less soluble casein; namely, particles of powdered isoelectric casein which went through the sieve No. 200. There is a sharp rise in viscosity in the first few minutes due to swelling of the micelles and this is followed by a rapid drop in viscosity due to the solution. In 4 cc. the drop in the curve is less steep because the casein is less rapidly soluble.

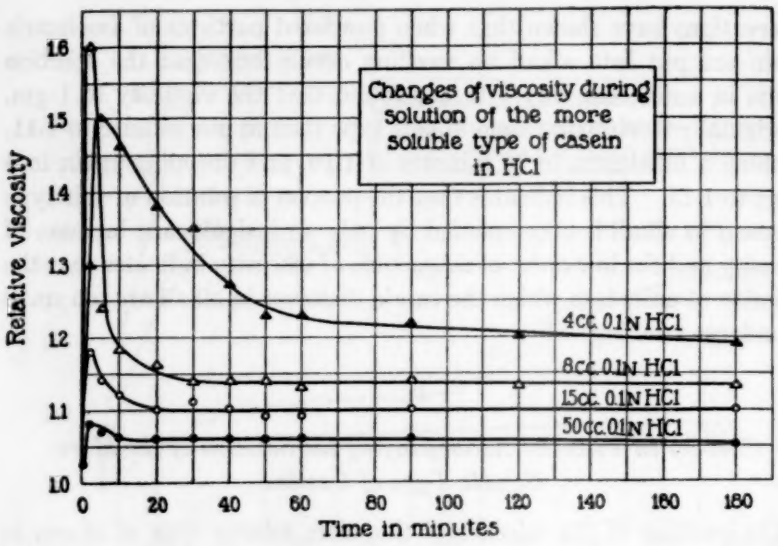

FIG. 5. Changes of viscosity accompanying solution of 1 gm. of powdered casein of the more soluble type in HCl.

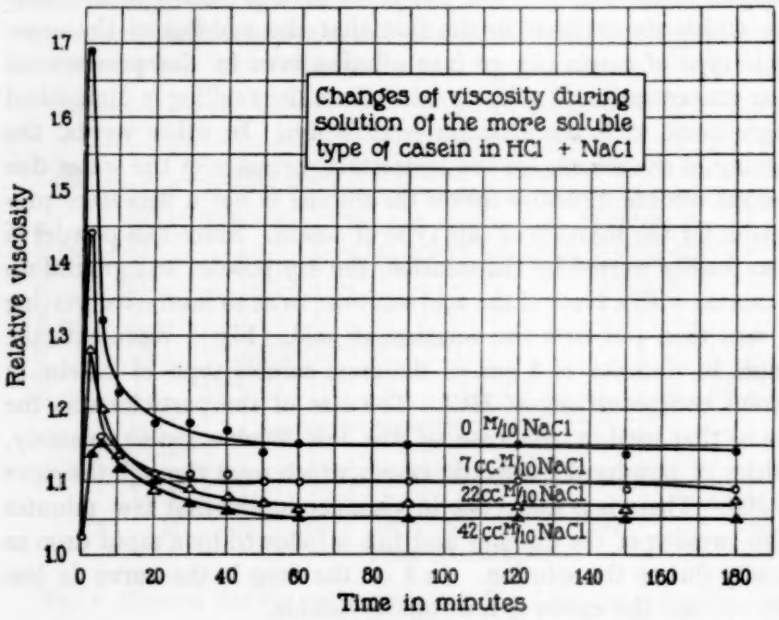

FIG. 6. Changes of viscosity accompanying solution of 1 gm. of the more soluble type of powdered casein in HCl solutions containing NaCl.

The rapid drop during the first 10 or 40 minutes, respectively, is followed by a very slow drop, the viscosity reaching in all solutions the value of 1.1 or less in the next 24 hours.

The presence of NaCl affects chiefly the degree of swelling and only to a slight extent (if at all) the rate of solution of the micelles of this type of casein. Hence the addition of salt only has the effect of lowering the viscosity (Fig. 6). The low order of viscosity in acid reached at the end of the experiment indicates that there are very few units left in the protein solution large enough to give rise to a Donnan equilibrium.

V.

The Ultimate Units in Casein Solutions in Acid.

The question now arises, what is the nature of the ultimate unit in casein solutions? 1 gm. of finely powdered casein, nearly isoelectric, going through the meshes of a sieve, No. 200, was put into 100 cc. of water containing various numbers of cc. 0.1 N HCl. The solutions were kept at 24°C. for 16 hours and were repeatedly stirred. After 16 hours, the volume occupied by the non-dissolved part of casein was measured in cc. The whole was then stirred up and the viscosity determined.

Fig. 7 contains the results of such an experiment with the less soluble type of casein. The abscissæ are the pH of the suspension or solution after 16 hours; for the convenience of the reader the cc. 0.1 N HCl contained in 100 cc. of casein solution at the beginning is written under the pH. The ordinates of one curve are those representing the relative viscosity, the ordinates of the other curve are the volume of the sediment in each solution, *i.e.* the undissolved part of the casein. Where the volume of the sediment was zero, all the casein was in solution or at least could not settle any more. This happened for the less soluble type of casein between pH of about 2.45 and about 1.85 (or in solutions which contained originally from 9 to 20 cc. 0.1 N HCl in 100 cc.). What interests us is the viscosity in this region of complete solution. It is above 1.1, thus indicating that a certain fraction of the 1 gm. of casein exists still in units large enough to give rise to a Donnan equilibrium and to undergo swelling. Before dis-

cussing this, it will be necessary to compare the curves for volume of sediment and for viscosity where the casein is not all dissolved. It is obvious that there exists a certain parallelism in the two sets of curves. At pH 4.2, the casein is insoluble and incapable of swelling and the viscosity is about 1.02. At pH 3.4 there is a slight amount of swelling, but the casein is still insoluble and both curves for volume of sediment and viscosity rise in a parallel way. At pH 3.0 the swelling increases and the values for viscosity and volume of sediment

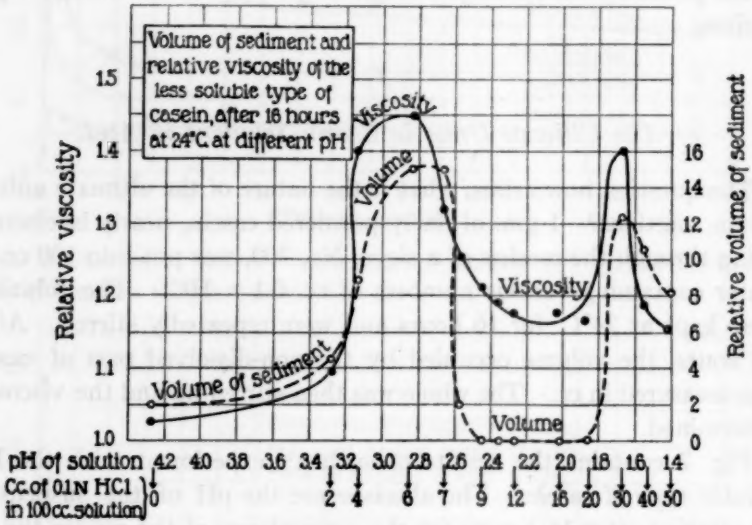

FIG. 7. Parallelism in value of sediment (or swelling) and viscosity of the less soluble type of casein in different concentrations of acid after 16 hours. Abscissæ, pH of casein suspension; ordinates of continued curve, viscosity; of interrupted curve, volume of sediment.

rise sharply, but the volume curve does not rise as steeply as the viscosity curve. This is due to the fact that some of the casein has gone into solution, thereby diminishing the volume of sediment. Between pH 2.7 and 2.6 there occurs a steep drop of volume of sediment, *i.e.* the whole gm. of casein goes into solution. There occurs also a steep drop of the viscosity curve but the drop is not so complete as the drop in the volume curve for the reason that some of the units of casein in solution are still in a state of swelling. At pH 1.8,

both curves rise again steeply because now the swelling though still considerable is not enough to permit the casein to go into solution. The reader will remember that unless a critical limit of swelling of the powdered particles of this type of casein is exceeded, the casein cannot be dissolved. At pH 1.8 the swelling is depressed in agreement with the theory of membrane equilibria. The relative viscosity will, however, be high since the relative volume of the particles in solution is high. At pH 1.6 and pH 1.4 the swelling will be more reduced on account of the Donnan equilibrium and the volume of sediment and the viscosity will drop.

The agreement between swelling of particles of casein and viscosity is so good that we have a right to apply this agreement to that part of the curve where the volume of the sediment is zero, *i.e.* where the whole casein is in solution. Figs. 1 and 3 show that with 8 or 9 cc. HCl in 100 cc. solution, *i.e.* at pH 2.5 or 2.4, the initial swelling and viscosity are enormous, the relative viscosity reaching a value of about 1.7, but that the viscosity drops rapidly. This can only be due to the fact that part of the casein is dissolved into units too small to give rise to a Donnan equilibrium. The viscosity drops finally to about 1.2. If all the casein in solution consisted of units too small to give rise to a Donnan equilibrium, the viscosity should on the basis of Einstein's theory have dropped to about 1.03 as it does in solutions of crystalline egg albumin, since the molar concentration and density of the 1 per cent casein solution was probably not very different from that of the 1 per cent solution of egg albumin. It is therefore possible to find out what percentage of the 1 gm. of casein is still in solution in the form of particles capable of giving rise to a Donnan equilibrium. These units must undergo the maximal swelling for a pH of 2.4. It was therefore necessary to find out what fraction of 1 gm. of the less soluble casein will give a maximal viscosity of about 1.2 at pH 2.45 at a temperature of 24°C. Fig. 1 shows that the maximal swelling is reached in about 30 minutes, which gives ample time to carry out the experiment. Doses of 0.25, 0.35, and 0.5 gm. of nearly isoelectric finely powdered casein of the less soluble type, going through mesh 200, were put into 100 cc. H_2O containing so much HCl that the pH of the casein mixture with acid was 2.45 in each case and the viscosity of each dose was measured in short intervals during the first 30 minutes at 24°C. The results are given in Table V.

The reader will see that 0.3 gm. of casein in 100 cc. solution of HCl, to give a pH of 2.45, has a viscosity of about 1.14, while the maximal viscosity of 0.5 gm. is above 1.2. We may therefore infer that at pH 2.45 more than one-half of 1 gm. of casein consisted of units too small to give rise to a Donnan equilibrium, while less than one-half of 1 gm. consisted of larger units.

The 1 per cent solutions of this type of casein are always slightly turbid, owing probably to the fact that they contain a number of

TABLE V.

Influence of Concentration of Casein in 100 Cc. HCl Solution at Point of Maximal Swelling, pH of Solution 2.45, on Relative Viscosity, T = 24°C. Time Experiment.

After minutes.	Gm. of casein per 100 cc. of solution.		
	0.25	0.35	0.5
1	1.005	1.008	1.016
5	1.008	1.046	1.066
10	1.025	1.060	1.084
15	1.055	1.085	1.192
20	1.060	1.110	1.235
25	1.096	1.118	1.260
30	1.092	1.140	1.280
35	1.100	1.135	1.245
40	1.085	1.135	1.230
45	1.092	1.125	1.210
50	1.085	1.118	
55	1.075		
60		1.110	
65	1.067		

small particles of insoluble denatured casein which, however, adds little to the viscosity.

Fig. 8 shows the curves for viscosity and volume of sediment after 16 hours at 24°C. at varying pH for the more soluble type of casein. 1 gm. of finely powdered casein (not quite isoelectric) of this type, going through mesh 200, was put into 100 cc. H_2O containing various numbers of cc. of 0.1 N HCl. At pH 3.0 and below all the casein went into solution. For this type of casein the solubility increases with the hydrogen ion concentration and no longer depends on preliminary swelling of the particles. Between pH 1.4 and 3.3 the viscosity curve

is a straight line, indicating that the viscosity is a linear function of the pH. This straight curve may be chiefly the expression of the Donnan effect on viscosity. The curves for the membrane potentials or osmotic pressure are also practically straight lines for the same pH interval. The viscosity value at pH 2.2 is 1.15 which is the same as that for the less soluble type of casein for the same pH. This would mean that the same relative proportion of casein, *i.e.* less than 0.5 forms units capable of giving rise to a Donnan equilibrium.

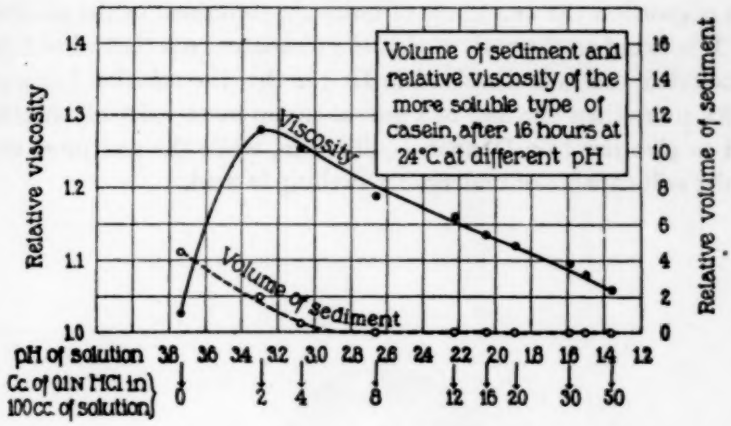

FIG. 8. Influence of pH on viscosity of solutions of the more soluble type of casein at different pH.

SUMMARY AND CONCLUSIONS.

1. The experiments show that suspensions of finely divided particles of insoluble proteins incapable of swelling in acid (denatured egg albumin, casein trichloroacetate, sulfate, etc.) raise the viscosity of the suspension but little and that the influence of acid on the viscosity is negligible.

2. The same is true for solutions of certain genuine proteins such as genuine crystalline egg albumin.

3. In contrast with these are proteins which swell in acid. It can be shown that where acid swelling of particles occurs, the viscosity is of a higher order of magnitude than where the swelling of particles is impossible and that the influence of acid on viscosity runs in these

cases parallel to the influence of acid on swelling. This is shown to be the case for casein in HCl.

4. It is shown that the swelling of the powdered particles which determines the high order of viscosity varies according to the theory of membrane equilibria.

5. These results are used to ascertain with the aid of viscosity measurements whether the ultimate units of genuine protein in *solutions* are aggregates large enough to give rise to a Donnan equilibrium; or whether they consist of particles below this limit; and in what porportion the two kinds of units are contained in the solution.

6. It is found on the basis of viscosity measurements that when 1 gm. of isoelectric casein is dissolved in HCl so that the solution has a pH of 2.45, more than one-half of 1 gm. of casein must exist as units too small to give rise to a Donnan equilibrium, while the rest must exist in units still capable of undergoing swelling in acid.